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FUNCTIONAL PROPERTIES OF NONHUMAN PRIMATE ANTIBODY TO PORPHYROMONAS GINGIVALIS

A
THESIS

Presented to the Faculty of
The University of Texas
Graduate School of Biomedical Sciences
At San Antonio
in Partial Fulfillment
of the Requirements for the Degreee of

MASTER OF SCIENCE

By
Dennis Michael Anderson

San Antonio, Texas
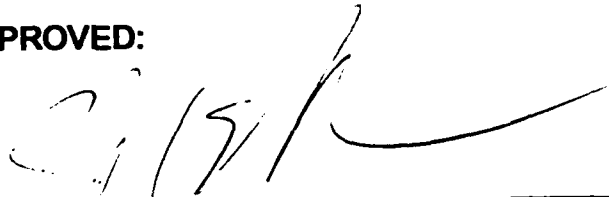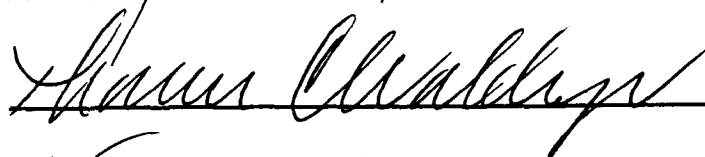
May 1993

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Dennis M. Anderson

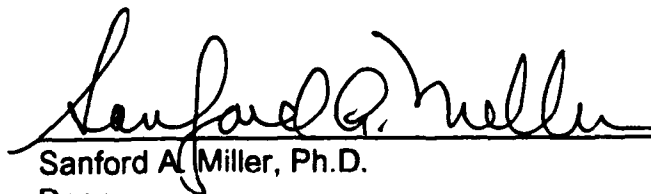**APPROVED:**

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DEDICATION

This thesis and research is dedicated to Terry, my wife and my two children, Grant age 4 and Adrienne age 3. It is only through their constant understanding and accompanying sacrifices that I completed such an involved research project. I will be forever indebted to them for this educational experience. I would especially like to acknowledge the extraordinary effort given by my wife who had to maintain a household and two small children by herself the majority of three years. To Grant and Adrienne, I hope that this thesis will one day encourage you to seek out and complete your dreams no matter how insurmountable and complex they may seem. I also hope that I can use the knowledge gained through your sacrifices to provide a better overall life experience and help you to achieve your full potentials. And lastly, I would like to dedicate the length and extensive intricacy of this project to my parents Leo and Barbara Anderson who were able to create and mold a person with enough self-control and concentration to complete such a project.

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FUNCTIONAL PROPERTIES OF NONHUMAN PRIMATE ANTIBODY TO *Porphyromonas gingivalis*.

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The nonhuman primate (NhP) serves as a useful model for examining the host-parasite interactions in *Pg*-associated periodontal disease. This study determined the influence of NhP sera on: (i) direct killing of *Pg* 3079.03, (ii) *Pg* induced superoxide anion (O_2-) release from human PMNs; and, (iii) the ability of PMNs to bind and phagocytize *Pg*. Three types of NhP sera were utilized: (i) normal/baseline (BAS), (ii) obtained after ligature induced periodontitis (LIG); and (iii) following active immunization with formalin killed *Pg* (IMM). Fluorescent dyes consisting of DAPI and propidium iodide were used to determine bacterial viability. Antibody mediated killing, and PMN attachment/ingestion of *Pg* were assessed by mixing sera, and *Pg*, or *Pg* and PMNs, noting the percentage of viable/nonviable cells, and the ratio of bound and phagocytized *Pg* to PMNs using fluorescent microscopy. O_2- production was quantitated spectrophotometrically by cytochrome c reduction and was used as an indirect measure of neutrophil activation. All assays were performed with/without the addition of human complement (C'). Significantly more direct killing occurred, in the absence of C', with both BAS and IMM sera as compared to the LIG sera ($p<0.03$). IMM sera in the presence of C' killed significantly more *Pg* ($p<0.04$). IMM sera incubated with *Pg*, in the presence of C', caused significantly greater ($p<0.01$) levels of

v

O_2- release from PMNs. Finally, it was noted that *Pg* remained viable after ingestion and did not kill the PMNs; and IMM sera significantly enhanced the uptake of *Pg* by the PMNs ($p < 0.009$). Active immunization with *Pg* elicited a functional antibody that enhanced direct killing, positively influenced the activation of PMNs, and enhanced the ability of PMNs to phagocytize *Pg*. Moreover, antibody produced as a sequela of progressing periodontitis appeared to lack these functions. Furthermore, a wide variability in functional capacity of the sera was noted, which may contribute to the susceptiblity to *Pg*, and this variability suggested that results from functional tests of serum antibody may aid in predicting host susceptiblity to disease and response to therapy.

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I. INTRODUCTION AND LITERATURE REVIEW

A.   Introduction

Periodontal disease is a multifactorial disease process that affects unique structures in the human body, the hard and soft tissues of the periodontium. The complexity is compounded by the presence of multiple putative pathogens (Moore, et al., 1982) and an intricate balance of host immune responses. The study of periodontal disease has evolved over the last century, from defining calculus as the primary etiologic agent, from the 1900's until 1965 when experimental gingivitis studies (Loe, et al., 1965) provided strong support for plaque as the primary etiologic agent. From that point, classification and enumeration of the subgingival microorganisms along with correlation studies of clinical parameters dominated the periodontal research arena through the 1980's.

The microbial data; however, did not reveal the key to better diagnostic and treatment modalities. Although some associations between localized juvenile periodontitis and *Actinobacillus actinomycetemcomitans* gained scientific support in a cause and effect relationship (Kornman and Robertson, 1985), not all localized juvenile periodontitis sites harbored *A. actinomycetemcomitans* (Mandell, et al., 1987). The treatment of localized juvenile periodontitis was modified to include antibiotic therapy (Kornman and Robertson, 1985), but overall, the treatment of adult periodontitis remained unchanged. The microbial studies failed to make a large impact on treatment modalities, leading researchers to concentrate next, on the host response.

The host response is an extremely complex and multidimensional biologic system providing protection for the host. To study the interaction between the host and a disease process, such as periodontal disease, one must begin approaching the investigation as a biologic process. The immune response must be divided into its

most basic components, then individual functions of each system should be defined, and algorithms proposed for the interactions between these defensive systems. It is only by investigating the specific interactions between the etiologic agents and the host, that we can hope to create better diagnostic parameters, and perhaps improve host defenses, as a method of eliminating disease progression.

B.   Immunology of Periodontal Disease

1.   Humoral Immunity

a.   Antibody

The immune system has been classically divided into two areas, the humoral response and the cellular response. These two systems have been shown to act both dependently and independently in host defense. The humoral response consists of two main components, immunoglobulins and complement. The immunglobulins in host defense are known as antibodies (i.e. IgG, IgA, IgM, IgD, and IgE). The early investigations of the immune response in periodontal disease attempted to determine the most basic of host responses by testing serum for antibodies against putative periodontal pathgens.

Courant and Gibbons (1967) were among the first investigators to establish that there was an antibody produced by the host which reacted with the lipopolysaccharide (LPS) from both laboratory strains of *Bacteroides*, and autologous strains of *Bacteroides* from the pockets of 16 patients with periodontal disease. The next study examined sera from healthy patients to determine if their sera had detectable levels of

antibody to periodontal pathogens that would react to laboratory strains of these putative pathogens (Hofstad, 1974). The results indicated that subjects free of periodontal disease produced a detectable level of antibody to several species of bacteria and that the antibody appeared to be directed toward the polysaccharide moiety of the lipopolysaccharide molecule.

Succeeding studies revealed that a host affected with periodontal disease could respond by generating higher serum levels of antibody, specifically IgG (Ebersole, et al., 1980, Ebersole, et al., 1986, Nakagawa, et al., 1990), to putative periodontal pathogens such as *A. actinomycetemcomitans* and *Porphyromonas gingivalis*. These immunglobulins were found to be specific for the organisms found in the periodontal pocket (Ebersole and Holt, 1988) and not just part of a non-specific host response.

The analysis of data gathered from serum antibody has been approached from two different avenues. Generally, the serum titers from the diseased patients are meaned and compared to mean serum levels from healthy patients. The diseased patients exhibit increased mean levels of antibody, to suspected pathogens, over healthy controls. One study demonstrated mean serum values of IgG specific for *P. gingivalis* approaching five times the amount found in controls (Mouton, 1985). However, when individual serum levels were analyzed independently, a great deal of variability existed, with up to half of the diseased population presenting with average to low titers compared to healthy subjects. Additionally, the clinical presentation gave no clue as to which subjects had either high or low serum levels of IgG. Another investigation; however, did show correlations between the anti-*P. gingivalis* IgG serum

level and a history of periodontal destruction, as detected by radiographic alveolar bone loss (Naito, et al., 1987).

Other investigators have examined the change in antibody levels associated with increasing age. Their data show that the overall level of total serum IgG increases with age for all patients, but that in patients with periodontal disease the level anti-*P. gingivalis* IgG was significantly greater than in healthy controls (De Nardin, et al., 1991). This led the authors to speculate on the meaning of this higher titer of serum IgG. The elevated titer of anti-*P. gingivalis* IgG may represent a predisposing factor for periodontal disease, or it may be that it is a result from periodontal infections in older adults.

Investigations looking even more specifically at IgG and its subclasses has shown that the mean levels of serum IgG1 and IgG2 were significantly elevated in adult periodontitis but that IgG4 may be a significant marker for patients with active disease (Wilton, et al., 1992). Others (Whitney, et al. 1992) have shown that in rapidly progressive periodontitis (RPP) patients, IgG2 was produced in higher quatities than IgG3, which was higher than either IgG1 or IgG4. Speculating on the functionality of these immunoglobulins based on the general properties of IgG subclasses, one might suggest that IgG2, which lacks strong complement fixation and opsonic properties, is not a very protective immunoglobulin, yet is produced in high amounts in diseased patients. The properties generally associated with IgG2 suggest that it is not a very protective antibody, yet in patients with community acquired pneumonia, low levels of IgG2 appears to be a marker of disease susceptabilty, and immunization to increase the level of IgG2 appears to be protective (Herer, et al., 1990).

Another area of investigation is that of avidity, defined as the strength of the bond between the antibody and its substrate. Some studies show this bond as stronger in the healthy patients, and weaker in patients with RPP, holding true for both the seropositive (high levels of IgG) and the seronegative (low levels of IgG) sera. However, in adult periodontitis, the IgG of diseased patients showed significantly greater binding to *P. gingivalis* than the healthy controls (Patters and Kornman, 1989). Following treatment; however, the avidity of the antibody has been found to increase, and may lead to an increase in the protective nature of the immunoglobulin (Chen, et al., 1991).

The treatment of periodontal disease appears to lower serum antibody levels, not only to putative pathogens but to species believed to be neutral in the disease process. A great deal of interpatient variation was found in serum antibody responses, sometimes without much difference in the clinical course of the disease (Aukhil, et al., 1988). There was also evidence that B-cells and plasma cells produce anti-*P. gingivalis* antibodies (Ogawa, et al., 1989) locally in the gingival tissues (Kagen, 1980) and that this level of antibody is higher than that found in serum. This local response was found to be greater in adults with untreated periodontitis as compared to adults following treatment, (Burstein, et al., 1987).

One study attempted to combine the above mentioned tests by looking at serum titer, avidity and subclass in adult patients with periodontal disease. They found 17 sera testing high in anti-*P. gingivalis* IgG via enzyme-linked-immunosorbant-assay (ELISA). Of these 17 only 3 sera were opsonic for a strain of *P. gingivalis* A7436, and it was only these same three that resulted in increased phagocytosis. The higher

level of IgG did not necessarily reflect a higher functional ability of the serum (Cutler, et al., 1991b). The authors speculated on the reason for this occurrance and felt that those three sera outperformed the other 14 because the antibody may have been reacting to a specific epitope on the bacterial surface, which may of been playing a role in antiphagocytosis. The binding of this site by antibody subsequently rendered the cell susceptible to ingestion by neutrophils.

The of role antibody in host defense for patients with periodontal disease (Genco, et al., 1974) has been speculated upon based on the general properties of antibodies and the potential interactions with host cells. These include both protective and potentially destructive responses toward host tissues. Activation of complement by antigen--antibody complexes, neutralization of bacterial allergins, toxins, or histolytic enzymes, and the enhanced opsonization or bacteriolysis of plaque bacteria are believed to be protective. The bactericidal activity of serum in combination with complement, appears to be limited when used *in vitro* against *P. gingivalis,* with direct killing decreasing as the number of bacteria used increased (Okuda, et al., 1986). The phagocytosis of antigen--antibody complexes through the interaction with Fc receptors on neutrophils appears protective, unless a subsequent release of lysozomes occurs, then the reaction may become destructive toward host tissues. Additional roles for antibodies include enhanced lymphoctye stimulation by antigen-antibody complexes and the release of lymphokines contributing to either protection, destruction or both. There is also the potential for a blocking of lymphocyte receptors by either free antibody or by antigen-antibody complexes leading to suppression of cell-mediated immune responses.

The literature describing the presence of antibody and its role in host defense is not clear at this time. The correlation between the disease process and the titer of antibody appears very weak. Most of the studies would agree that there is a great deal of inter-patient variation in the production of immunoglobulin and that this variability makes it hard to draw clear conclusions from such a complex system. The data from testing avidity, subclassification and titer are important, but do not go far enough in defining the properties of the serum antibody and predicting its role in host defense. It is only by determining the true functionality of the serum immunoglobulins that we can hope to define the role of these proteins in host defense and predict disease outcome.

b.   Complement

Complement was the name given to a single compound in serum that worked in combination with antibodies to agglutinate and lyse red blood cells. Complement actually consists of 11 discrete serum proteins that can act alone or in combination with immunoglobulins to amplify the immune response. There are two proposed pathways for complement to interact with target cells: the classical complement cascade; and the alternative complement system. The classical pathway is dependent upon immunoglobulins and C1, C4, and C2 to activate C3 which then initiates a common cascade active in both pathways, the activation of C5, C6, C7, C8, and C9. The alternative complement system activates C3 independent of interactions with either immunoglobulin or the C1-C4-C2 complex. The major proteins released upon cleavage of basic

complement activation, include C3a and C5a which have chemotactic properties, and C3b which renders the target cell more susceptible to phagocytosis by leukocytes.

The cleavage of complement may serve an important immunopathologic mechanism in the development of gingival inflammation. The cleavage products from complement factors C3, C4 and B are higher in gingival crevicular fluid of gingivitis patients as compared to healthy controls (Henry, et al., 1987, Patters, et al., 1989). The contribution of complement in phagocytosis studies reveals that it is an important adjunct to antibody dependent phagocytosis (Cutler, et al., 1991a). Based on the general known properties of complement in conjunction with these data, it suggests that complement contributes significantly to the serum opsonic capacity in the gingival crevice and the release of these complement cleavage products serve to increase the local tissue inflammation.

There are three main receptors for complement on host cells, known as CR1 (CD35), CR2 (CD21) and CR3 (CD18). The CR1 receptors have been identified on erythrocytes, B lymphocytes, a subset of T lymphocytes, monocytes, and macrophages, neutrophils, eosinophils, glomerular podocytes, and follicular dendritic cells. This receptor is believed to interact primarily with C3b and C4b. The receptors on these cells in their resting states will only express 5% to 10% of their CR1 sites on the plamsa membrane, but such agents as C5a, formy-methionyl-leucyl-phenylalanine (f-MLP), endotoxin, certain cytokines, calcium ionophores, and phorbol esters will upregulate the CR1 receptor increasing the number of sites available. CR1 receptor regulates the

alternative pathway activation via three possible mechanisms, and the classical pathway via four possible mechanisms (Ahearn and Fearon, 1989) by interfering with the availabilty of key proteins in each pathway.

CR2 receptors bind the cleavage products of C3 and C4 and are mainly limited to B lymphocytes, human thymocytes, pharyngeal and cervical epithelium, and follicular dendritic cells. The receptor, in addition to its complement site, is also a receptor for the Epstein-Barr virus (Horejsi, 1991). This receptor may participate in the regulation of T cells, and antigen-independent stimulation, for B cell proliferation. It has also been shown to help regulate the complement cascade by serving as a cofactor (Ahearn and Fearon, 1989).

The last major receptor is CR3 which is stucturally different from the other two receptors. It belongs to the leukocyte integrin family and is present on neutrophils. Its major role is in adhesion of myeloid cells to opsonized particles (Horejsi, 1991).

Complement and its specific receptors serves as an integral part of both the humoral and cellular host systems contributing to both potentially protective and destructive reactions. Further elucidation of the complement components and its receptors will hold the key to further defining the intricate functions of the immune system.

2. Cellular Immunity

The cellular arm of host defenses is comprised of a number of cells specifically neutrophils, macrophages, T lymphocytes and B lymphocytes. Of most importance in periodontal disease is believed to be the neutrophil, commonly called the first line of defense in the periodontal pocket. The importance of the neutrophil in periodontal disease comes from the severe periodontal involvement in diseases deficient in neutrophil function such as Down's Syndrome (Cohen, et al, 1961), Chediak-Higashi Syndrome (Lavine, et al., 1976), Diabetes mellitus (Glickman, 1972), Lazy Leukocyte Syndrome (Miller, et al., 1971), and Leukocyte Adhesion Deficiency (Waldrop, et al.,1987). From the study of these conditions, deficiencies in neutrophil function have left the host open to severe periodontal involvement.

In order for neutrophils to function properly they must perform a number of functions such as adherence to the vascular endothelium, migration beyond the wall of the blood vessel and enter the focus of inflammation to begin phagocytosing the pathogenic agents or releasing defensive agents. Any deviation from normal can leave the host susceptible to disease. The reason for deviation from normal may be intrinsic within the neutrophil such has been found in the majority of cases in 86% of a juvenile periodontitis group presenting with chemotactic defects (Lavine, et al., 1979). In rapidly progressive peridontitis (RPP) patients, only 48% had detectable leukotactic defects with the majority associated with defects in the serum (Lavine, et al., 1979). Another study looking only at patients within one family with RPP, found both adhesiveness and the phagocytic index at a lower level of adhesiveness than controls. The adhesiveness returned to near control level in the presence of autologous serum

suggesting that the defect was intrinsic and that the serum had additional factors present to help compensate for this defect. The phagocytic capacity of these patients remained below that of the controls in all conditions (Gutierrez, et al., 1991). Conclusions from these studies are that both defects in adhesion to the vessel wall, and in phagocytosis were present in this group of RPP patients.

The chemotactic response of neutrophils may be elicited directly from chemotactic substances in plaque bacteria such as bacterial antigens, and endotoxin, or secondarily from bacterial products promoting chemotactic factors from the activation of complement.

There are two major killing mechanisms associated with neutrophils: oxidative and nonoxidative. Nonoxidative killing takes place in the abscence of oxygen and is mediated by a variety of factors including lysosomal enzymes, peptides, cationic proteins, defensins and lactoferrin. Oxidative killing is dependent upon the presence of dioxygen (O_2) and the formation of toxic oxygen metabolites such as H_2O_2, O_2^-, and hydroxyl radicals. Another important component of the oxidative system is myeloperoxidase which is found in the azurophilic granules of neutrophils and contributes to the formation of hypochlorous acid which acts as a potent antimicrobial oxidant (Miayasaki, et al., 1986).

Assessment of the nonoxidative functions of the neutrophil can be assessed *in vitro* by using phagocytic assays to evaluate the ability of the neutrophil to attach and ingest the target organism (Cutler, et al., 1991b). For evaluation of the oxidative functions, and indirectly the interaction of the neutrophil with the bacteria *in vitro*, the quantitation of superoxide produced from peripheral neutrophils is used (Van Dyke, et al., 1988).

C. *Porphyromonas gingivalis* in Periodontal Disease

1. Virulence Factors

P. gingivalis is a gram-negative, strictly anaerobic, nonmontile, nonsporeforming rod shaped bacteria that produces brown to black pigmented colonies when grown on blood containing media. This organism has long been associated with areas of periodontal destruction (Slots, 1979a) and has become the focus of many microbial studies in periodontitis. The virulence associated with species is not uniform across all strains (Neiders, et al., 1989) since those strains producing infection, seem to possess specific characteristics allowing evasion of the host defense mechanisms (Van Winkelhoff, et al., 1988).

P. gingivalis has been studied in the hopes of discovering the factors that enhance the potential virulence of this bacteria. In order to be effective in a dynamic environment such as the gingival sulcus an organism must have an effective form of adherence. Two adhesins on the cell's surface probably aid in attachment to both host cells and other bacteria: a heat stable, thin, curly filament known as fimbriae, and a separate surface protein responsible for hemagglutinating activity. The outer layer associated with some of the more virulent strains is known as a capsule. This structure is high in polysaccharide and is believed to contribute to blocking phagocytosis by neutrophils (Okuda and Takazoe, 1973). Beneath the capsule in gram negative bacteria, is the cell wall containing a complex molecule, lipopolysaccharide (LPS). The LPS of *P. gingivalis* differs from other gram negative bacteria not only in structure but function with the ability to stimulate bone resorption, inhibit collagen formation (Miller, et al., 1986) and induce interleukin-1 (Hanazawa, et al., 1985).

While bound LPS in the cell wall adds virulence to gram negative bacteria, some *P. gingivalis* strains have the ability to package this product as outer membrane vesicles. These appear to be extensions of the cell wall, containing LPS, toxins and enzymes, which pinch off into the environment. Vesicles appear to have a number of functions. Pretreating human serum with *P. gingivalis* vesicles has been shown to inhibit the killing activity of serum towards *P. gingivalis* as well as other bacteria such as *Capnocytophaga ochracea* (Grenier and Belanger, 1991). The ability to remove antibacterial elements from serum may be an important element in infection, allowing the expression of otherwise nonvirulent organisms, which are normally cleared by serum, to become infectious (Ingham, et al., 1977). It may be just such a mechanism at work in periodontal disease where such a large number of species are present.

This bacterial species is also effective in neutralizing humoral elements of host defense, with trypsinlike proteases that degrade IgG, IgM, (Grenier, 1992) IgA, IgD, IgE (Gregory, et al., 1992) and complement factors C3, C4, and B (Schenkein, 1988). Hoover et al. (1992) has proposed that it may be the trypsinlike protease sites responsible for the associated haemagglutinating properties of *P. gingivalis*. This organism appears to be well equipped to disarm the non-oxidative mechanisms of neutrophil defense as well, by inactivating cathepsin G, elastase, bacterial-permeability increasing factor and defensins (Odell, 1992). Research also shows bacterial supernatants of *P. gingivalis* may inhibit the migration of neutrophils and moncytes (Fotos, et al., 1992), by porin-like activity causing a depolarizing of neutrophil membranes decreasing their response to chemotactic peptides (Novak and Cohen, 1991). Not only is this species able to evade nonoxidative and phagocytosis type defenses, but Amano et al. (1992) has shown it does well against the oxidative mechanisms as well by

production of a superoxide dismustase neutralizing the free oxygen radicals produced by neutrophils.

2. Prevalence in Periodontitis

The crevicular microbiota in humans undergoes a shift from gram positive cocci and rods during health to gram negative motile and nonmotile rods, with increasing inflammation, as in severe gingivitis and adult periodontitis. A significant percentage of this increased gram negative population has been identified as *Poryphyromonas gingivalis* (Slots, 1977, White and Mayrand, 1981, Zambon, et al., 1981). Several studies have found strong correlations between the presence of *P. gingivalis*, and elevated gingival inflammation scores and deeper periodontal probing depths. Another strong positive correlation was shown between a loss of periodontal attachment and the presence of *P. gingivalis* (Slots, et al., 1985), *Actinobacillus actinomycetemcomitans, P. gingivalis* and *Provetella. intermedia* (Brag, et al., 1987), or *Wolinella recta (Campylobacter rectus), P. intermedia, Fusobacterium nucleatum, P. gingivalis,* and *Bacteroides forsythus* (Dzink, et al., 1988). In gingival health *P. gingivalis* or the above listed combinations were found to be absent, leading some to suggest the eradication of this bacteria as a therapeutic endpoint.

The strength of the above listed studies, trying to correlate disease activity with the presence of certain bacteria, rests on the sensitivity of accurately detecting specific species from subgingival plaque samples. With the advent of DNA probe technology, which tests for the presence of specific DNA sequences unique for that species of bacteria, sensitivity of bacterial detection has increased. One study using the DNA probe detected *P. gingivalis* in 91% of adult patients with periodontal destruction (Haffajee, et al., 1992). The use of

DNA probes appears to be more sensitive than culturing methods in detecting *P. gingivalis*. Loesche, et al. (1992) found 82-88% of sites harbored *P. gingivalis* by DNA probe analysis in periodontal sites of destruction, where culturing methods found only 42% of those samples *P. gingivalis* positive. The DNA probe has also been used to determine the incidence of *P. gingivalis* associated with age. The data revealed that *P. gingivalis* was more prevalent in patients over thirty, and rarely found in younger patients, where *A. actinomycetemcomitans* was more prevalent in the younger age groups and diminished with increasing age (Savitt and Kent, 1991). The use of this new technology may increase the correlative relationships between disease and health by revealing the true nature of the subgingival microbiota.

Another new technique for differentiating between strains of bacteria within one species is DNA fingerprinting. The use of this technique in examining subgingival species of bacteria revealed that most patients appear to be infected exclusively by one clonal type of *P. gingivalis* (Loos, et al., 1992) with only a few patients harboring two or more clonal types. This means that if an attempt in the future is made to modify the host response against *P. gingivalis*, only one strain may need to be targeted to get the desired effect.

D.   Nonhuman Primate as a Model for Periodontitis

Periodontal disease, due to its chronic nature, lacks a definitive clinical indicator of disease activity (a gold standard). This has increased the complexity of studying this disease process, often requiring extended periods of time to insure the loss of periodontal attachment. The use of human subjects in studies examining disease

progression without treatment, is not ethically acceptable; therefore, the search for a suitable animal model has insued. A number of animal models such as rodents, sheep, cats, dogs, swine pigs and nonhuman primates have been investigated. None of the models have produced a consistent pattern of spontaneously occurring periodontal disease comparable to humans (Page, 1988). Therefore, one method has been to select an animal model with clinical, microbiological and immunological responses as close to humans as possible using a ligature to induce periodontitis. Ligature induced periodontitis in the nonhuman primate appears very similar but not identical to human periodontitis, and appears to be the best model available.

1. Clinical

The nonhuman primate or more specifically the Old World monkeys belonging to the species *Macaca* appear to be the animals of choice since their teeth and periodontium closely resemble that of humans. The monkeys are large, and their usually docile nature allows dental procedures to be carried out with only mild sedation. The intraoral structures are of sufficient size to allow manipulation of the periodontium with standard instrumentation and procedures (Krygier, et al., 1973).

There is a naturally occurring gingivitis present in captive *Macacae*, with associated plaque and calculus, which resolves following plaque and calculus removal. Increasing plaque levels with subsequent gingivitis was induced in animals by stopping oral hygiene measures and placing them on a soft diet. Their rate of inflammation was slightly more rapid than that seen in humans.

Their natually occurring gingivitis appeared histologically identical to that found when gingivitis was induced by a silk ligature (Nalbandian, et al., 1985). Acute exacerbations of gingivitis with a silk ligature can lead to destructive episodes of periodontitis in four to six weeks (Kiel, et al., 1983), with associated epithelial ulceration and osteoclastic bone loss (Heijl, et al., 1976). Brecx, et al. (1985) ligated teeth in this animal model and found a dense buildup of bacteria with an associated apical migration of the junctional epithelium, a loss of connective tissue collagen, and a loss of connective tissue attachment. Osteoclastic bone resorption was also noted around the ligated teeth. Wirthlin and Hussain (1992) reported smaller changes in attachment levels with ligature induced periodontitis than those found in humans, but felt these to be substantial differences for the smaller monkey teeth. Heijl, et al. (1976) noted that a partial repair of a bony lesion may follow an episode of bone loss with the establishment of a stable lesion in the squirrel monkey.

Clinically normal monkey gingiva was found to be histologically indistinguishable from that of human tissue. During a state of gingivitis a widening of intracellular spaces in the crevicular epithelium occurred with an infiltration of the gingiva by neutrophils, lymphocytes and plasma cells. A disorganization of the collagen matrix was also noted (Krygier, et al., 1973).

2. Microbiology

The microbiota of the nonhuman primate has been studied in health, and in stages of gingivitis and periodontitis by a number of researchers. Cock and

Bowen (1965), and Slots and Hausmann (1979), found the indigenous flora of ligature induced periodontitis similar to that found in humans. Kornman, et al. (1981) followed the progression of disease from health through four stages of disease, monitoring the oral microbiota. They found a shift in subgingival organisms similar to that found in humans. A dominantly gram positive flora was present in health, and shifted to a primarily gram negative flora in disease, dominated by *Bacteroides asacchrolyticus (P. gingivalis)*. Slots (1979a) found a similar increase in the presence gram negative organisms in disease, with *Bacteroides melaninogenicus* strains increasing in proportion from a few percent in health to approximately 66% in areas with periodontitis.

Holt et al. (1988) strengthened the relationship between *Porphyromonas (Bacteroides) gingivalis* and the clinical characteristics of periodontitis by implanting a rifampin resistant strain of nonhuman primate *Bacteroides* into a naturally occurring ligature induced subgingival flora which then resulted in a burst of alveolar bone loss. Thus, the emergence of *P. gingivalis* in the subgingival plaque was capable of producing clinical signs of periodontitis, with associated alveolar bone and clinical attachment loss.

3. Immune Response

Studies investigating the complex response of interactive host defense systems collectively known as the immune system have been initiated for the nonhuman primate relative to periodontal disease. Manti, et al., (1984) looked at the cellular response of T and B lymphocytes in the cynomolgus monkey as

chronic gingivitis converted to progressing periodontitis. Active periodontitis was associated with an increase in *B. gingivalis*, a decrease in the T-cell helper/suppressor ratio, and an increase in B-cell levels. Following induction of periodontitis an immunomodulating drug, thymopentin, was used to initiate phenotypic and functional maturation of the immature T-cells. This resulted in an increase in the helper/suppressor T-cell ratio and decreased the *P. gingivalis* levels, suggesting a role for T-cells in modulating this disease.

The humoral response has also been investigated in this animal model establishing relationships between bacteria, immunoglobulin and clinical responses. Clark, et al. (1988) and Holt, et al. (1988) were able to establish an association between increased levels of Black-Pigmented *Bacteroides* (BPB) species in the gingival crevice and increased levels of serum antibody to this group of bacteria. Another group (Mc Arthur, et al., 1989) working with squirrel monkeys sought to establish a relationship between serum anti-*P. gingivalis* IgG levels post immunization (with *P. (Bacteroides) gingivalis*) and the level of BPB levels in the gingival crevice. As IgG increased after immunization the level of BPB decreased. A similar study with *Macacae* monkeys (Nisengard, et al., 1989) was performed by immunizing with *Bacteroides macaca* (a monkey strain of BPB). They found that immunization increased the serum anti-*B. macacae* IgG level and that this appeared to have a protective response by decreasing the number of *B. macaca* in the pocket and decreasing the amount of bone loss that occurred compared to nonimmunized controls.

Ebersole, et al. (1991) immunized a group of female macaque monkeys with formalin killed bacteria. This resulted in a 2 log increase in IgG, IgM, and IgA levels specific for the homologous bacteria used for immunization. Like the above study, the elevated immunglobulins appeared to significantly inhibit the

emergence of homologous organisms in the gingival crevice. However, an associated effect on slowing disease progression was not seen; some of the immunized animals responsed with increased levels of bleeding on probing and loss of attachment, compared to sham immunized controls. This suggests other mechanisms of disease progression may be at work such as a hypersensitivity reaction toward multiple bacterial antigens in the subgingival plaque.

The nonhuman primate does not have spontaneous periodontal disease identical to that of humans; however, this animal appears to be the best model available at this time due to its established similarities in the clinical, microbiological and immunological responses to periodontal disease. Some of the basic questions regarding disease progression in the nonhuman primate have been answered in order to establish this as an animal model. However, many questions still remain, especially regarding the intricacies of the host--parasite relationship. Further research in the area of immunology for this animal model will help to clarify not only its relationship in periodontal disease but the complex interactions of an immune response in disease.

E.   Statement of Problem

This study will characterize the functional activity of nonhuman primate antibody derived from 3 model systems, (natural, immunized and ligature) to *Porphyromonas gingivalis.* This study will attempt to answer the following questions.

1. Does antibody containing serum, alone or when combined with complement, have a direct killing effect on *P. gingivalis*?

2. Does antibody containing serum, alone or when combined with complement, generate superoxide release from human neutrophils in the presence of *P. gingivalis*?

3. Does antibody containing serum increase the functional activity of human neutrophils for ingestion and killing of *P. gingivalis*?

There are three major hypotheses addressed in this study. The first to be tested is that nonhuman primate serum containing antibody following immunization will directly kill *P. gingivalis*. The second hypothesis is that nonhuman primate serum containing antibody induced following immunization will induce the release of superoxide from human neutrophils in the presence of *P. gingivalis*. The third hypothesis is that nonhuman primate serum containing antibody following immunization will increase the functional activity of human neutrophils with increased attachment and ingestion of *P. gingivalis*.

II. MATERIALS AND METHODS

A.   Experimental Protocol

Serum samples were obtained from animals entered into in the following protocol. Twenty adult female cynomolgus monkeys of the species *M. fascicularis* were used. Baseline sera, for determination of natural antibody levels, were drawn from weeks 0-5. Five animals were administered an active immunization with formalin killed *P. gingivalis* 3079.03 cells fixed in 0.5% buffered formalin-saline, stored in phosphate-buffered saline containing 1 mM EDTA, and emulsified with an equal volume of incomplete Freund's adjuvant (IFA; Difco) (Ebersole, et al., 1991). Each animal was immunized with 10^9 total *P. gingivalis* cells or with a placebo (phosphate-buffered saline in IFA). The immunizations were administered in three seperate injections separated by 2-week intervals, starting at week 10 and ending at week 14. At 16 weeks a ligature was placed around three adjacent teeth in the mandibular left quadrant with 3-0 silk sutures. Serum samples were then drawn by venipuncture at 16 through 51 weeks. The ligature and immunized serum samples used in this study were collected between 22 and 27 weeks (Figure 1). All serum samples were heated at 56°C for 30 minutes in a water bath to inactivate any residual complement in the nonhuman primate (Nhp) serum.

B.   *Porphyromonas gingivalis* 3079.03

P. gingivalis 3079.03 was grown in an anaerobic atmosphere of 80% N_2, 10% CO_2, and 10% H_2. It was routinely maintained on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) with 5% sheep's blood and supplemented

Figure 1 Nonhuman Primate Experiment Protocol. This figure represents the experimental protocol using *Macaca fasicularis* as the animal model, immunized with 10^9 *P. gingivalis* 3079.03 as outlined. Serum was drawn for the natural, immunized or ligature groups as illustrated at 0-5 or 22-27 weeks. Ligature induced periodontitis was initiated at 16 weeks in the mandibular right quadrant.

Nonhuman Primate Experimental Protocol

Animal: *Macaca fasicularis*

Bacteria: *Porphyromonas gingivalis 3079.03*

Ligature placed

0   5   10   12   14   16   22   27

WEEKS

☒ **Natural Serum Drawn**

▬ **Immunization with 10^9 *P.g***

▭ **Test or**

Control Serum Drawn
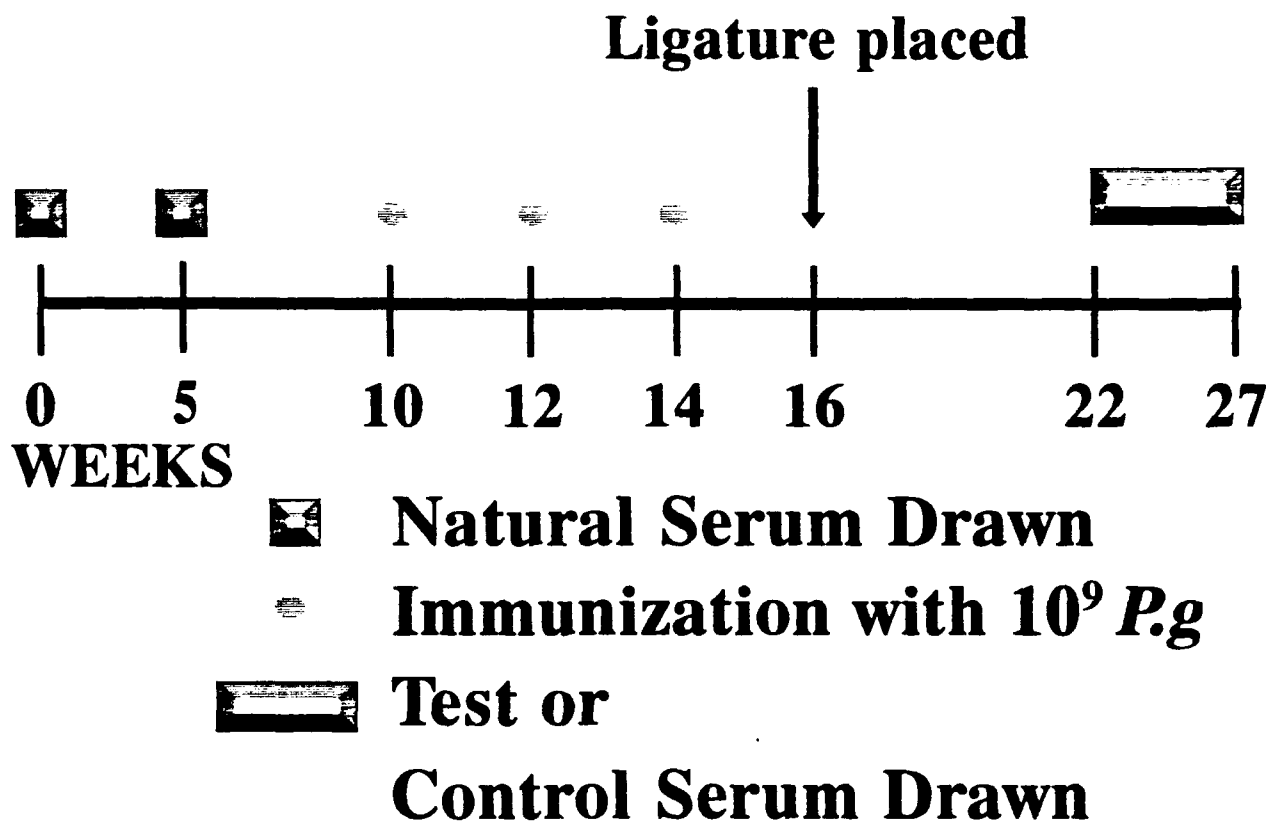
with 5 µg/ml of hemin and 1 µg/ml of menadione, with reculturing performed at weekly intervals.

For antigen preparation and viability assessment, the organisms were grown in Mycoplasma Broth Base (MBB) supplemented with hemin (5 µg/ml) and menadione (1 µg/ml) under anaerobic conditions at 37°C. Each liter of media was prepared by dissolving 21 grams of Mycoplasma Broth Base into 1 L of distilled water. To that, 2.5 ml of hemin stock solution (1mg/ml) was added. The solution was mixed, autoclaved for 30 minutes and allowed to cool. Two ml of filter-sterilized menadione (0.5 mg/ml) was added to the solution. The media was kept at 37°C for 48 hours to check for contamination. It was subsequently aliquoted into sterile glass tubes (5ml/tube) and stored under anaerobic conditions.

The enriched MBB solution was inoculated with *P. gingivalis* 3079.03 and incubated for 48 hours. During late log phase the bacteria were quantitated by spectophotometric absorbance at 660 nm, (Spectronic 710, Bausch & Lomb) with absorbance approaching 1.0 (4 x 10^9 bacteria/ml). The approximate number of bacteria were enumerated from an absorbance curve generated for *P. gingivalis* in mycoplasma broth (Figure 2).

C. ELISA for IgG

The level of antibody specific for *P. gingivalis* 3079.03 in the non-human primate sera was quantitiated by an enzyme-linked immunosorbent assay (ELISA) using formalin killed *P. gingivalis* 3079.03 cells. The absorbance values obtained were compared to a reference standard prepared by hyperimmunization of three nonhuman primates with bacteria (Ebersole et al., 1991). The serum titers were converted to ELISA units using linear regression analysis.

Figure 2 Growth Curve for *P. gingivalis* in Mycoplasma Broth. This graph represents the relationship between the absorbance vaiue of a bacterial suspension on the y-axis and the number of *P. gingivalis* as represented on the x-axis. The absorbance was recorded at 660 nm. The E in the x-axis labels stands for exponent.

Growth Curve for P. gingivalis in Mycoplasma Broth

Absorbance

Number of P. gingivalis per ml

Absorbance at 660 nm
E=exponent
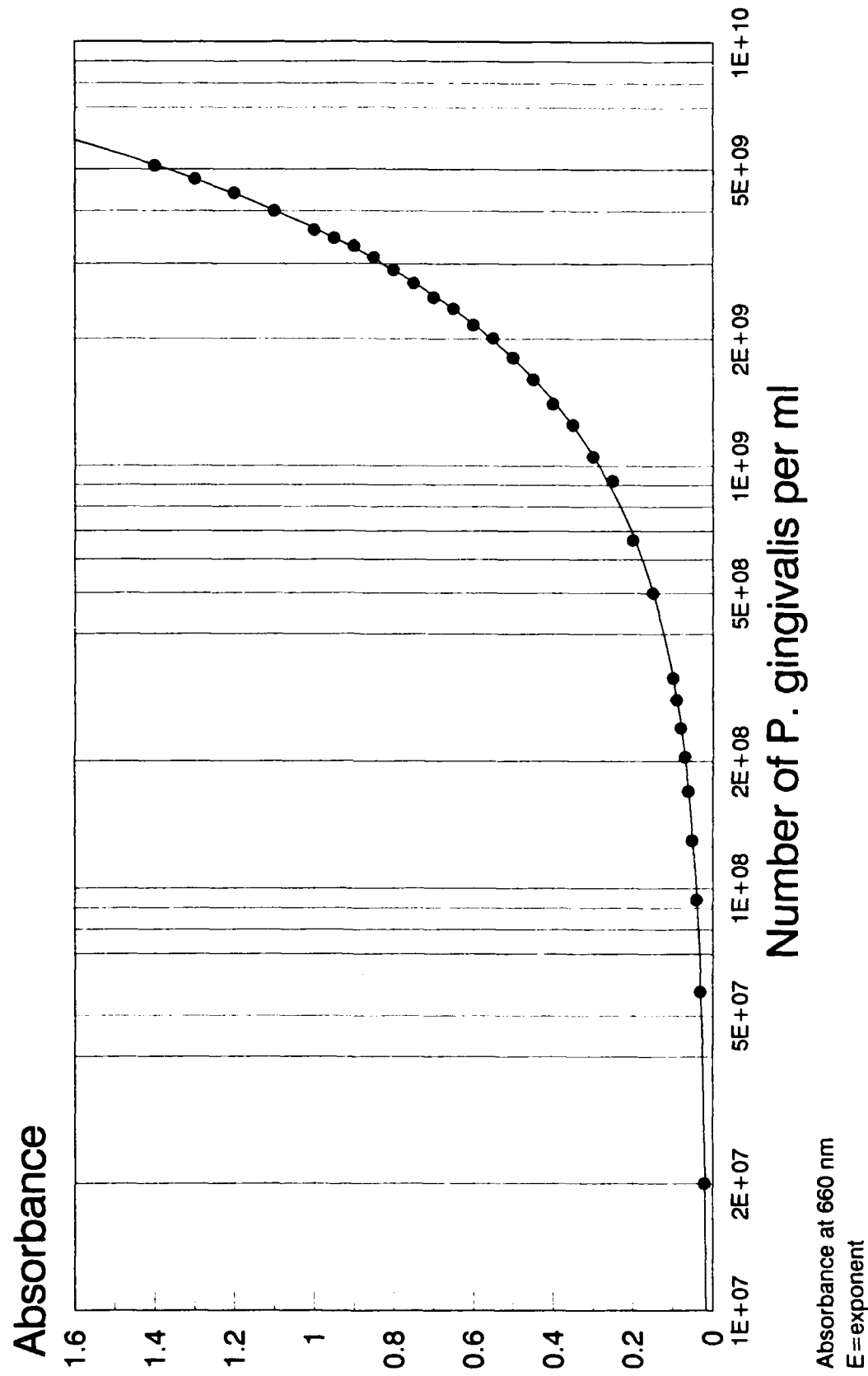
Determination of antibody titers specific to *P. gingivalis* 3079.03 in the sera were performed to determine the source of complement (human or rabbit) that contained the least amount of anti-IgG to *P. gingivalis* 3079.03, as well as to estimate the level of IgG, IgM, and IgA antibody to *P. gingivalis* 3079.03 in the Nhp sera. The amount of specific IgG, IgM or IgA to *P. gingivalis* 3079.03 was determined by absorbance values at 410 nm (Biomek 1000, Beckman)(Table 1).

D. Complement Fixation Test

The complement titer was quantitiated using a complement fixation test (Palmer and Whaley, 1986). This test required the determination of optimal hemolysin concentration, followed by experimentation to determine the highest dilution of serum containing a level of complement that would produce 100% hemolysis of sheep red blood cells. Stock reagents were prepared for use, (Appendix A1) along with sheep red blood cells in Alsever's solution (Appendix A2). Complement containing sera were prepared from three different sources (Appendix A3) and stored at -70°C until use. Preparation of a hemoglobin color standard was used to standardized the assay using absorbance at 540 nm (Appendix A4). Sheep red blood cell sensitization with hemolysin (i.e. anti-sheep red blood cell antibody) was carried out using a standard protocol (Appendix A5).

1. Titration of hemolysin

The optimal dilution of hemolysin (rabbit anti-sheep RBC, Pel-Freeze) was determined prior to testing the complement activity level. To accomplish this a series of hemolysin dilutions were created in Barbital-buffered diluent (BBD), (1:1000, 1:1500, 1:2000, 1:2500, 1:3000, 1:4000, 1:8000). Sheep red

TABLE 1

ELISA Units of Antibody to *P. gingivalis* 3079.03

GROUP	Ig Class							MEAN	STD DEV
ANIMAL #		R23	R23	P63	P61	R19	P69		
Natural	IgG	23.84	18.13	11.49	8.5	3.02	3.31	11.17	(7.69)
Natural	IgM	3.48	1.54	0.7	0.45	0.7	0.05	1.03	(0.94)
Natural	IgA	12.65	6.97	6.67	0.17	0.14	0.02	4.17	(4.56)
ANIMAL #		P65	R65	R61	P87	P83	R67		
Immunized	IgG	1306.4	568	528	387.2	317.28	234	556.67*	(354.21)
Immunized	IgM	60.72	13.26	29.65	29.19	7.24	102.21	40*	(32.41)
Immunized	IgA	30.98	175.77	393.04	15.03	1.52	263.18	192.67*	(145.47)
ANIMAL#		R05	R23	M99	P99	P97	N11		
Ligature	IgG	60.09	36.5	23.5	6.5	6.2	3.38	22.50	(20.78)
Ligature	IgM	8.49	4.97	6.12	7.47	2.84	0.2	4.83	(2.67)
Ligature	IgA	2.71	20.32	0.75	1.57	1.09	0.05	4.28	(7.09)

* Immunized sera contains significantly more IgG, IgM and IgA
than the Baseline and Ligature sera at p<0.005.

blood cells were then sensitized with each respective dilution of hemolysin and allowed to incubate for 15 mintues in a 37°C water bath. Two sets of 7 tubes were labelled with the above dilutions. Each tube received 0.4 ml of cold BBD, 0.4 ml of either 1:5 or 1:10 dilution of rabbit complement and 0.2 ml of the respective sensitized sheep red blood cell suspension containing hemolysin. The mixture was gently vortexed and incubated for 30 minutes at 37°C in a water bath, shaken gently, and incubated for another 30 minutes.

Following incubation the samples were centrifuged at 600 x g for 5 minutes (Sorvall RT6000B, Dupont) at 4°C. The samples were read at 540 nm (Beckman DU-65 Spectromphotometer Instrument) using BBD solution as a blank. An optimal dilution of 1:1500 for hemolysin was determined. (Table 2).

2.   Titration of complement

Commercially available complement is commonly supplied as whole serum from both rabbit and human sources. To determine the titer of complement present in these sera, dilutions of both rabbit and human whole sera were prepared using BBD, (1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320). Sheep red blood cells sensitized with a 1:1500 dilution of hemolysin were added to each of these dilutions. The samples were placed in a 37°C water bath for 30 minutes, shaken, and returned to the water bath for another 30 minutes. Following this, the samples were centrifuged for 5 minutes at 600 x g at 4°C. Absorbance values were determined at 540 nm and compared to a linear graph of the standard. (Appendix A4). A percentage of hemolysis was determined for rabbit complement and 2 human sources of complement. (Table 3).

After determining the complement titer level of the rabbit whole serum, a pilot study was performed using a 1:10 dilution of rabbit serum, a 1:10 dilution of

Table 2

Determination of Optimal Hemolysin Dilution

1:5 Complement Dilution

Dilution of Hemolysin	1:1000	1:1500	1:2000	1:2500	1:3000	1:4000	1:8000
Absorbance	1.077	1.068	1.088	1.060	1.089	1.045	0.969
% Hemolysis	100	100	100	100	100	100	100

1:10 Complement Dilution

Dilution of Hemolysin	1:1000	1:1500	1:2000	1:2500	1:3000	1:4000	1:8000
Absorbance	0.843	0.704	0.555	0.456	0.460	0.282	0.199
% Hemolysis	100	87	67	55	57	32	21

Table 3

Determination of Optimal Complement Dilution

Rabbit Complement

Dilution of Complement	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Absorbance	0.832	0.190	0.049	0.031	0	0	0
% Hemolysis	100	20	0	0	0	0	0

Human Sigma Serum (39 CH50 units/ml)

Dilution of Complement	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Absorbance	1.077	1.079	1.045	0.990	0.524	0.194	0
% Hemolysis	100	100	100	100	64	20	0

Human Complement (DMA)

Dilution of Complement	1:10	1:20	1:40	1:80	1:160	1:320	1:640
Absorbance	1.391	1.323	1.218	1.319	0.747	0.137	0.013
% Hemolysis	100	100	100	100	93	12	0

Determination of optimal complement dilution was performed with hemolysin concentration of 1:1500 dilution. The percent of hemolysis was determined from the hemoglobin color standard (Appendix A4).

heat inactivated (56°C for 30 minutes) rabbit serum and a 1:10 dilution of 5% bovine serum albumin (BSA control) in a direct killing assay against *P. gingivalis* (Section E below). This study was performed to prevent the introduction of experimental error from the complement source. The results of this study showed that the rabbit serum alone had significantly higher levels of direct killing against *P. gingivalis* than either the heat inactivated rabbit serum or the BSA control (Figure 3). These results suggested the possiblity that the rabbit whole serum contained antibody to *P. gingivalis.*

To test this possibility an ELISA was performed on each source of human and rabbit sera according to a standardized ELISA protocol. (Appendix B). Based on the absorbance values, the human serum DMA contained the least amount of IgG specific to *P gingivalis* 3079.03, and could effectively function as a source of complement at a dilution of 1:100. (Figure 4). The pilot study testing whole serum as a complement source in a direct killing assay against *P. gingivalis* was repeated with both DMA whole serum and heat inactivated serum. The results showed that the DMA serum had no more killing activity against *P. gingivalis* than the BSA control (Figure 5).

E.   Direct Killing Assay

1.   DAPI staining protocol (Figure 6)

Verification of uniform morphology and Gram stain was accomplished using darkfield microscopy and Gram stain techniques prior to manipulation of the bacteria. *P. gingivalis* was harvested in late log phase. The bacterial suspension, in mycoplasma broth, was placed into 1.5 ml plastic microcentrifuge tubes (1.5 ml, 39 mm x 10 mm dia., Sarstedt, Germany) and centrifuged

Figure 3 Killing of *P. gingivalis* by Rabbit Complement. This graph represents the percentage of *P. gingivalis* killed when interacted with either heat inactivated rabbit serum diluted 1:5 (Heat Inactivated C'), rabbit serum diluted 1:5 (Rabbit C' 1:5) or rabbit serum diluted 1:10 (Rabbit C' 1:10). The reagents were incubated for 30 minutes at 37°C on a tilting table.

**Killing of P. gingivalis
by Rabbit Complement**

Percent of P.g. killed

50

40

30

20

10

0

Heat Inactivated C'   Rabbit C' 1:5   Rabbit C' 1:10
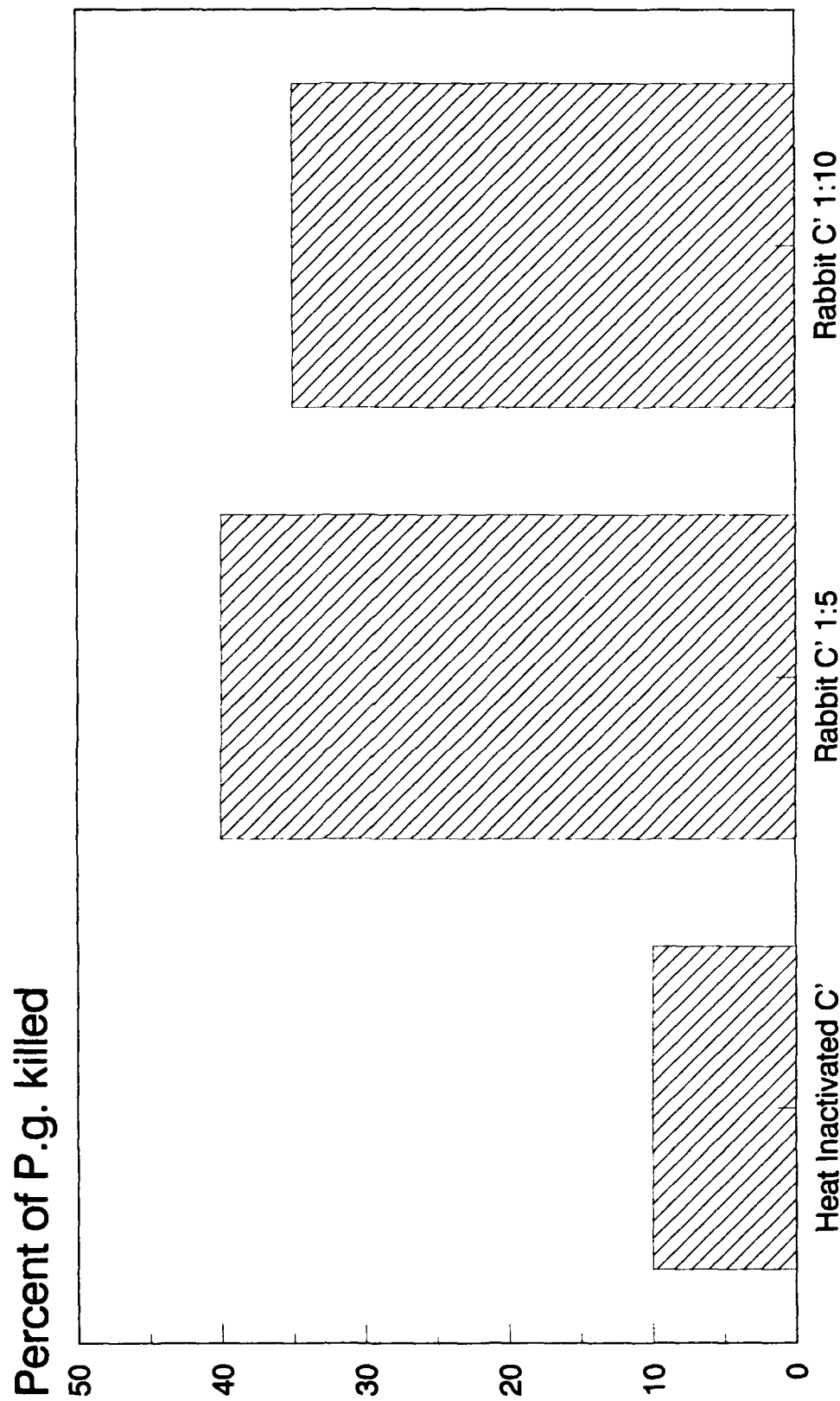
P.g. and Test Solution Incubated for
30 minutes at 37C on tilting table

Figure 4 Amount of IgG to *P. gingivalis* 3079.03 in Rabbit and Human Serum
Determined by ELISA. This graph represents the relative amounts of IgG in five sera:
commercially available human serum (Human SIGMA), human donor DMA (Human
DMA), human donor JLE (Human JLE), human donor HC (Human HC), and
commercially available rabbit serum from Pel-Freeze (Rabbit PF). The relative
absorbance of these sera is represented on the y-axis and the dilution of the sera on
the x-axis.

Amount of IgG to P. gingivalis 3079.03 in Rabbit and Human Serum Determined by ELISA
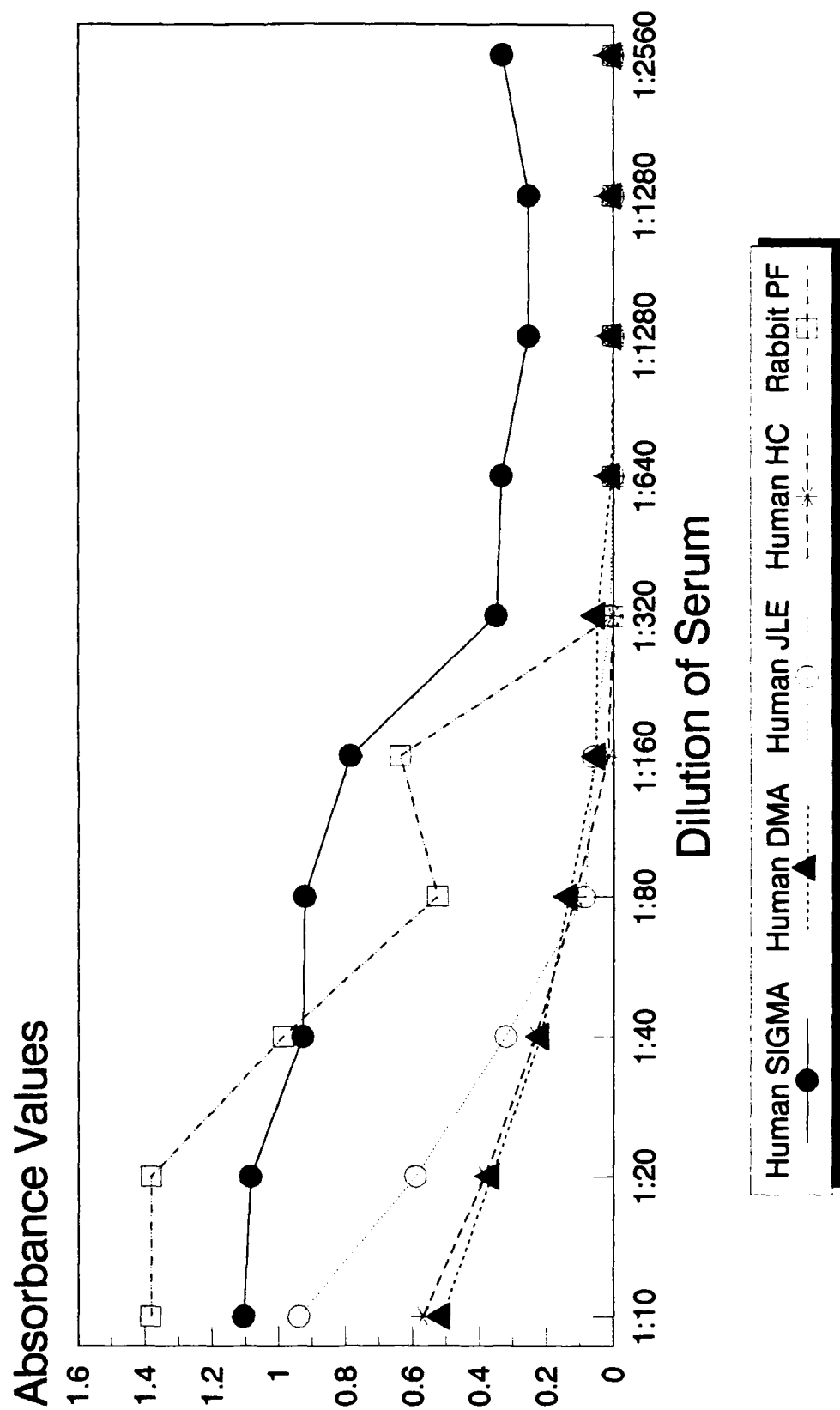
Figure 5 Killing of *P. gingivalis* by Human Complement. This graph represents the percentage of *P. gingivalis* killed on the y-axis, when incubated with either heat inactivated DMA serum diluted 1:100, DMA serum diluted 1:100, or 5% bovine serum albumin (BSA) 1:100 as labelled on the x-axis. The reagents were incubated for 30 minutes at 37˚C on a tilting table.

Killing of P. gingivalis
by Human Complement

Percent of P.g. killed

Heat Inactivated DMA serum 1:100   DMA Serum 1:100   BSA 5% 1:100

100
80
60
40
20
0
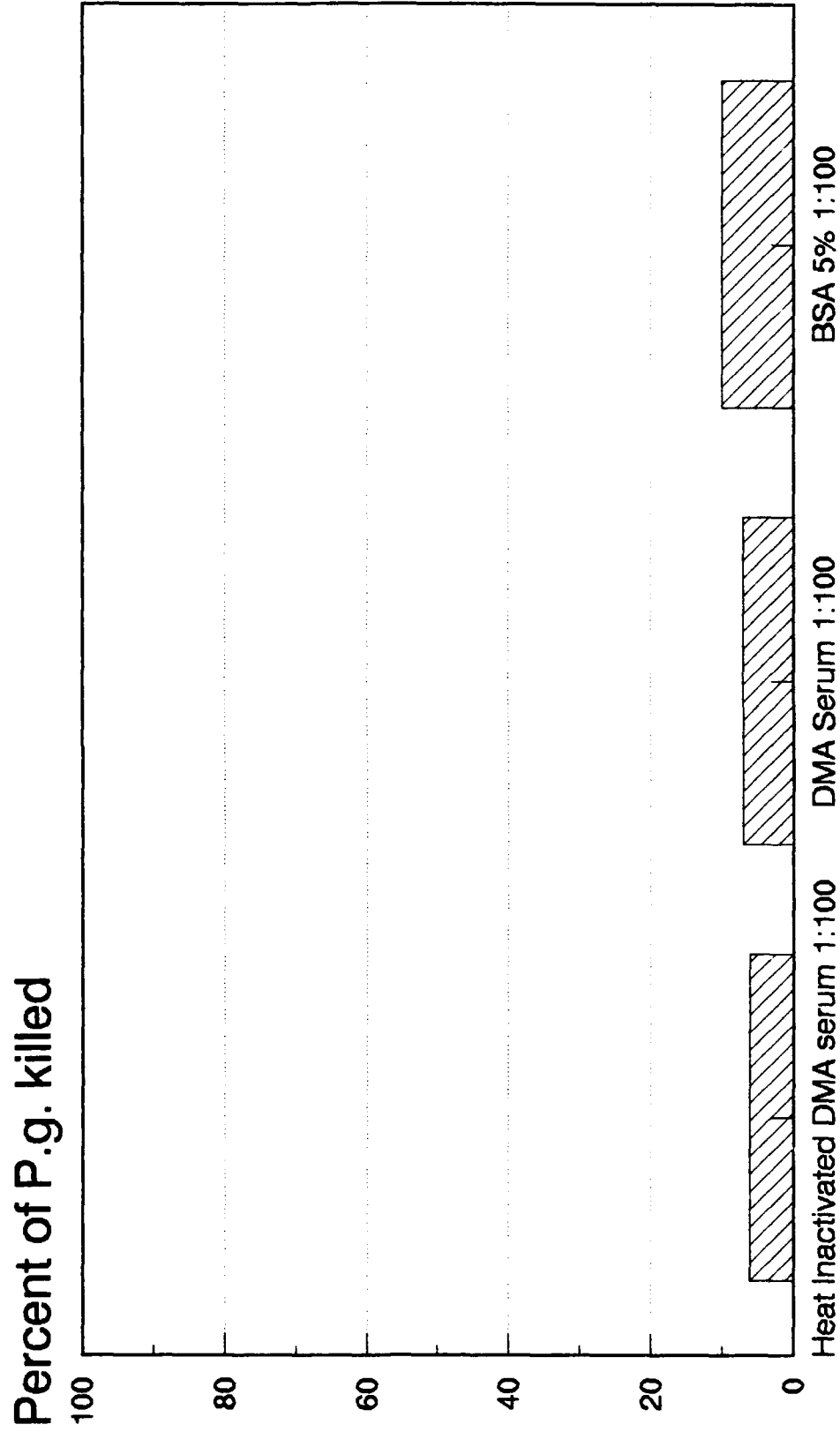
P.g. and Test Solution Incubated for
30 minutes at 37C on tilting table

Figure 6 Protocol for Staining with DAPI. This figure outlines the procedure to stain *P. gingivalis* with the fluorescent dye DAPI.

Protocol for Staining with DAPI

Read OD of *Pg* in broth at 660nm/Centrifuge 5 minutes at 6000 rpm

→

Remove Supernatant and add DAPI (10μg/ml), Vortex,
Incubate 10 minutes

→

Wash Procedure (x3): Centrifuge 5 mintues at 6000. Remove
supernatant. Add BBD (Buffered Barbital Diluent) and Vortex.

→

Remove Supernatant. Add BBD and Sonicate for 2x 5 seconds.

→

DAPI staining is complete.

(Microcentaur Microcentrifuge, Fisons) on high for 5 minutes. The supernatant was removed and 200 µl of DAPI (4'-6-diamidino-2-phenylindole.2HCl, Sigma Chemical) (10 µg/ml in distilled H_2O) was added, vortexed (VWR, Vortexer 2, Scientific Industries, Inc.) on high and incubated for 10 minutes at room temperature essentially as described by Cutler, et al. (1991a). The solution was centrifuged for 5 minutes on high and the supernatant removed. A series of three washes was performed using BBD (Barbital-buffered diluent) as follows; 200 µl of BBD was added and vortexed until the bacteria were well suspended; the tubes were centrifuged on high for 5 minutes, and the supernantant was removed. This was repeated 2 more times. Next, 1 ml of BBD was added to the pellet and sonicated (Sonifier, Cell Disruptor 200, Branson) in an ice bath at output-3, duty cycle 40%, continuously, for two 5 second periods. The absorbance of the suspension was again determined at 660 nm to quantitate the number of bacteria present after staining with DAPI (Figure 2).

2. Direct killing assay (Figure 7)

A direct killing assay was performed in a total volume of 1 ml. To accomplish this 500 µl of DAPI stained P. gingivalis suspension was added to either 10 µl of Nhp sera, 10 µl Nhp sera plus 10 µl of complement, or 10 µl of 5% bovine serum albumin (control) and the total volume brought to 1 ml using BBD. The solution was sonicated for two 5 second periods and incubated on a tilting table at 37°C for 30 minutes. The solution was sonicated again following incubation for two 5 second periods. To stop the reaction, the sample was diluted 50% by combining 500 µl of chilled BBD with 500 µl of test solution. Finally, 5 µl of propidium iodide (500 µg/ml in PBS) was added. The samples were stored in an ice bath until read.

Figure 7 Direct Killing Assay Using DAPI and Propidium Iodide. This figure outlines the procedure used to conduct the direct killing assay incubating *P. gingivalis* with experimental reagents.

Direct Killing Assay Using
DAPI and Propidium Iodide

P. gingivalis Stained with DAPI

↓   ↓

Nonhuman Primate Serum   Nonhuman Primate Serum
(1:100 Dilution)   (1:100 Dilution)
                                    PLUS Complement (1:100)

↓   ↓

Incubation for 30 minutes at 37°C/Sonicate

↓

Add Propidium Iodide. Remove
5µl, place on slide with cover slip.

↓

Enumerate Blue (vital) and Red (non-vital)
P.g. under appropriate
fluorescent filtration.
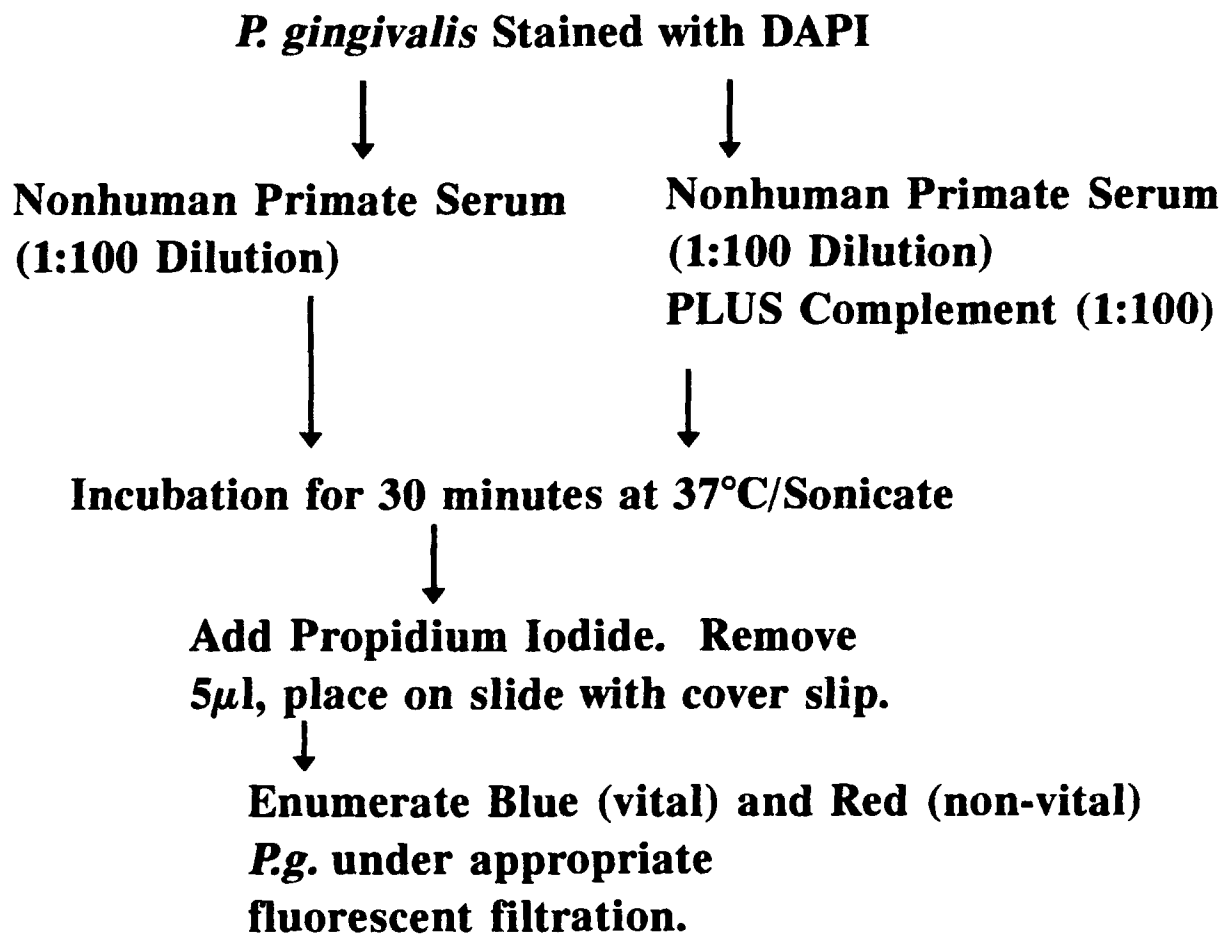
A sample was analyzed by placing 3.5 µl of the suspension on a freshly cleaned glass slide and covering with a coverslip. After the coverslip was seated, the slide was held motionless for 2 minutes before being read under 1000x. Three slides for each sample were created and ten consecutive fields for each slide were counted, giving a total of 30 microscopic fields for each sample.

The samples were read with Type A emersion oil on a Vanox T (Olympus) microscope equiped with fluorescence. To enumerate DAPI stained (vital) cells the U filter was used, and for propidium iodide stained (non-vital) cells, the G filter was used (Appendix C). All samples were run against a control of 5% bovine serum albumin (BSA) (Sigma Chemical).

F.   Preparation of Human Neutrophils

Human neutrophils were prepared for assay using a Ficoll centrifugation method (Boyum 1968). Standard concentrations of reagents (Appendix D) were used throughout the procedure. The neutrophils were harvested fresh each morning, stored on ice and used as soon as possible after preparation.

Neutrophil preparation began by drawing 40 ml of whole blood by venipuncture from healthy, non-smoking adult volunteers. Six ml of acid citrate dextrose (ACD) was added to the whole blood to act as an anticoagulant and mixed well. Ten ml of 6% dextran solution was then added. The red blood cells (RBC) were allowed to settle to the bottom of the syringe for 15 minutes or until there was a distinct line between the buffy coat and the RBCs. The buffy coat was removed and equally divided into two 50 ml plastic centrifuge tubes and the total volume brought to 40 ml with cold PBS with gentle mixing. The specimens were centrifuged at 580 x g for 10 minutes at 4°C (CR422, Jouan). The supernatant was removed and the pellet gently vortexed. Next, 3 ml of cold PBS was added to each tube and vortexed. This solution was gently layered

onto 3 ml of Ficoll Paque solution (Pharmacia LKB) and centrifuged at 1000 x g for 30 minutes at room temperature. The supernatant was suctioned off and the sides of the plastic tube wiped with two sterile cotton tip applicators. The remaining pellet contained neutrophils and some red blood cells (RBCs). The RBCs were lysed by vortexing the pellet, adding 10 ml of dH_2O to the pellet and gently mixing for 15 seconds. At the end of 15 seconds 500 μl of 20x saline was added to the solution to normalize the molarity of the dH_2O. The mixture was again centrifuged at 580 x g for 10 minutes at 4°C. The supernatant was removed and the pellet resuspended in a few drops of PBS and gently vortexed. The total solution was brought to 2 ml and the number of PMNs determined using a Coulter Counter ZM. A normal yield from 40 ml of whole human blood was 7-10 x 10^7 PMNs/ml and was routinely 96-97% pure PMNs. The PMN suspension was stored on ice until use.

G.   Superoxide Anion Assay (Figure 8)

The amount of superoxide released from human PMNs was determined by measuring the change in absorbance of cytochome C compared to a control and estimating the nanomoles of superoxide released. Following an acid wash (dichromate acid) of the cuvettes the working solutions (Table 4, Appendix D) were added in the following order: (i) cytochrome C; (ii) cytochalasin B; (iii) superoxide dismutase (SOD) (controls only); (iv) Krebs Ringer Phosphate plus glucose (KRPg); (v) PMNs (1 x 10^6 cells); (vi) Nhp serum (1:100 dilution); (vii) human serum containing complement (1:100 dilution); and, (viii) P. gingivalis 3079.03 (4 x 10^8 washed 3 times in PBS). The ratio of PMNs to P. gingivalis was approximately 1:400. To record changes in absorbance the cuvettes were loaded into a spectrophotometer (Lambda 6 UV/VIS Spectrophotometer, Perkin Elmer) which automatically subtracted the absorbance of the control cuvette (mixture containing SOD) from the experimental mixture using a

Figure 8 Biochemical Assay of Superoxide Anion Production. This figure outlines the protocol used to measure the superoxide production released from human neutrophils under different experimental conditions.

Biochemical Assay of Superoxide Anion Production

TEST

| Cytochrome C |
| Cytochalasin B |
| KRPg |

Neutrophils + Pg
1:400 ratio
Nhp Serum

Neutrophils + Pg
1:400 ratio
Nhp Serum + Complement

| Cytochrome C |
| Cytochalasin B |
| *Superoxide* |
| *Dismutase* |
| KRPg |

Neutrophils + Pg
1:400 ratio
Nhp Serum

Neutrophils + Pg
1:400 ratio
Nhp Serum + Complement

CONTROL

30 Minute
Incubation → Absorbance
Recorded

Test ABS - Control ABS =
Net Cytochrome C
Reduction

Calculations Performed
and Stored in Computer

Table 4

BIOCHEMICAL ASSAY OF SUPEROXIDE ANION PRODUCTION

Superoxide assay reagents added to cuvettes for PMNs at 5.6×10^7 in PBS and *P. gingivalis* 3079.03 at 4×10^9 in PBS in the order listed in the table from left to right. Amounts given in µl.

SAMPLE	Test Control	CYTO-C	CYTO-B	SOD	KRPg	PMNs	Ab	C'	*Pg*
Pg alone	T	100	5	—	795	—	—	—	100
	C	100	5	20	775	—	—	—	100
PMNs alone	T	100	5	—	877	18	—	—	—
	C	100	5	20	857	18	—	—	—
Pg + PMNs	T	100	5	—	777	18	—	—	100
	C	100	5	20	757	18	—	—	100
Ab + PMNs	T	100	5	—	867	18	10	—	—
	C	100	5	20	847	18	10	—	—
C' + PMNs	T	100	5	—	867	18	—	10	—
	C	100	5	20	847	18	—	10	—
C'+Ab+PMNs	T	100	5	—	857	18	10	10	—
	C	100	5	20	837	18	10	10	—
Pg+Ab+PMNs	T	100	5	—	767	18	10	—	100
	C	100	5	20	747	18	10	—	100
Pg+C'+PMNs	T	100	5	—	767	18	—	10	100
	C	100	5	20	747	18	—	10	100
Pg+C'+Ab+PMNs	T	100	5	—	757	18	10	10	100
	C	100	5	20	737	18	10	10	100

CYTO-C	Cytochrome C
CYTO-B	Cytochrome B
SOD	Superoxide Dismutase
KRPg	Krebs Ringer Phosphate plus glucose
Ab	Nonhuman Primate Serum
C'	Human Serum Containing Complement
Pg	*P. gingivalis* 3079.03

microcomputer and specialized software (Softways Lambda 4 Software Ver. 4.36). The solutions were allowed to react for 30 minutes.

Three seperate donors supplied PMNs on three different days. Each of the 18 Nhp sera were run once each day with and without human serum containing complement. The sequence of testing the Nhp sera was systematically changed each day to control for any decrease in PMN activity over time. The absorbance values were stored electronically until data analysis was performed. The nanomoles of superoxide were calculated using the following formula:

nanomoles of superoxide = Abs_E -- Abs_I / 0.021

where, Abs_E = Ending Absorbance; Abs_I = Initial Absorbance; and 0.021 = nanomolar extinction coefficient for cytochrome C (Cohen and Chovaniec, 1978). Superoxide production and release was expressed as nanomolars/10^6 PMNs

H.   Ingestion, Attachment and Killing Assay

1.   Ingestion and attachment assay

This protocol was used to quantitate the number of P. gingivalis that were either ingested by or attached to PMNs. The following solutions (Appendix D) were added to 250 µl polyethylene tubes (35 mm x 6 mm dia.) in this order: (i) KRPg (Krebs Ringer Phosphate plus glucose); (ii) propidium iodide; (iii) N-ethymaleimide (NEM)(controls only); (iv) PMNs (1 x 10^6 in PBS); (v) serum containing complement; (vi) Nhp serum; and (vii) P. gingivalis 3079.03 (1 x 10^8 bacteria) (Table 5, Figure 9). The ratio of PMNs to P. gingivalis was approximately 1:100. The tubes were incubated for 15 minutes in a 37˚C waterbath followed by storage in an ice water bath.

Table 5

INGESTION, ATTACHED AND KILLING ASSAY

Ingestion, attachment and killing assay reagents added to the polyethelene tubes for PMNs at 3.6 x 10^9 in PBS and *P. gingivalis* 3079.03 at 1 x 10^8 in PBS in the order listed in the table from left to right. Amounts given in µl with a total volume of 200 µl.

SAMPLE	NEM	PI	PMNs	KRPg	Ab	C'	Pg
PMNs alone	—	2	10	188	—	—	—
PMNs+Pg	—	2	10	160	—	—	28
PMNs+Pg+NEM	2	2	10	158	—	—	28
PMNs+Pg+C'	—	2	10	158	—	2	28
PMNS+Pg+Ab	—	2	10	158	2	—	28
PMNs+Pg+Ab+ NEM	2	2	10	156	2	—	28
PMNs+Pg+Ab+C'	—	2	10	156	2	2	28
PMNs+Pg+Ab+C'+ NEM	2	2	10	154	2	2	28

PI	Propidium iodide
NEM	N-ethylmaleimide
KRPg	Krebs Ringer Phosphate plus glucose
Ab	Nonhuman Primate Serum
C'	Human Serum Containing Complement
Pg	*P. gingivalis* 3079.03

Figure 9 Neutrophil Ingestion and Killing of *P. gingivalis*. This figure diagrams the protocol used for the attachment, ingestion and killing assays as human neutrophils were incubated with *P. gingivalis* under various experimental conditions.

Neutrophil Ingestion and Killing of
P. gingivialis
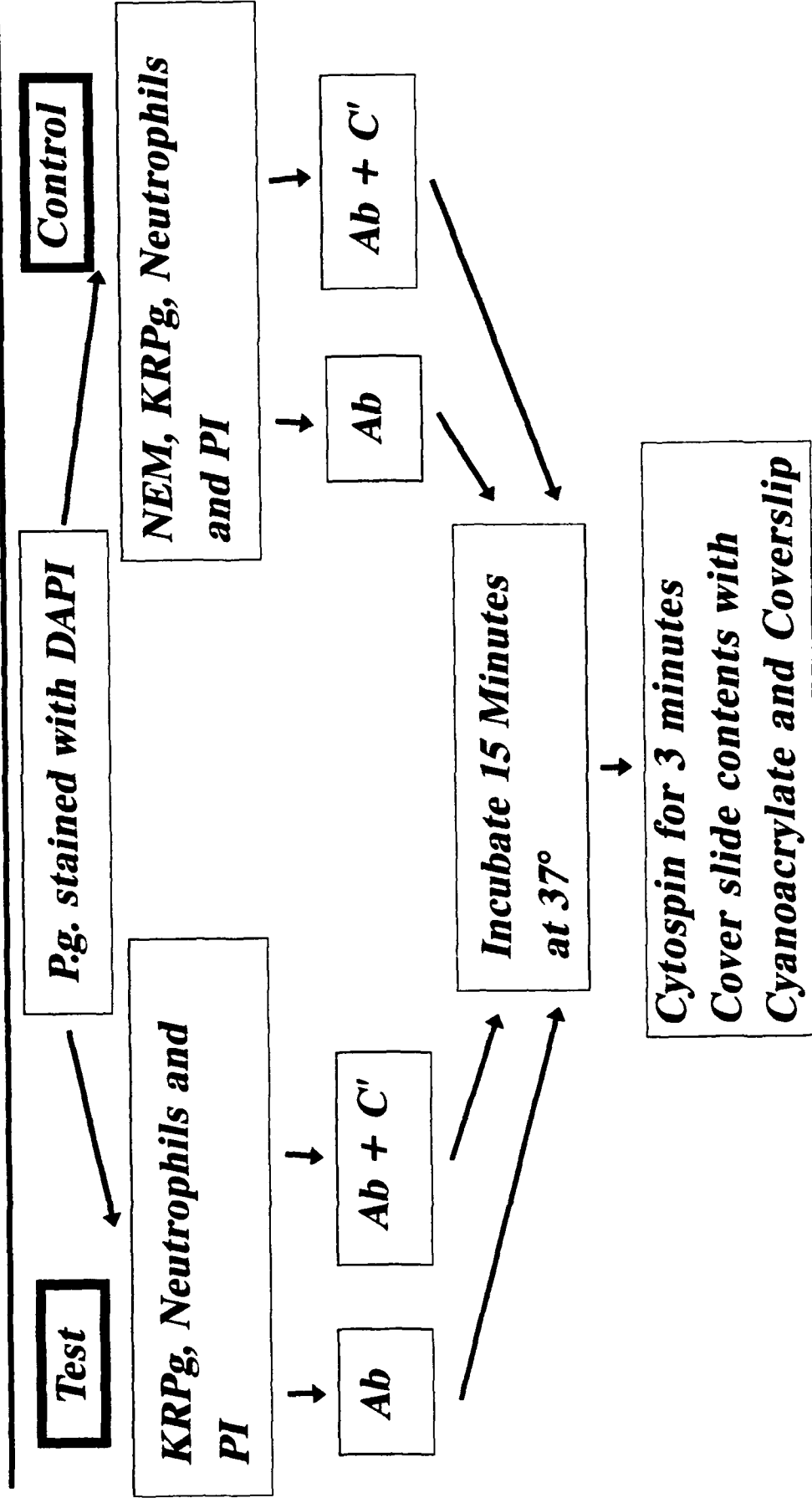
The following procedure was used to preserve the specimens for future quantitation. Each plastic retainer in a Cytospin 2 centrifuge (Shandon) was prepared with 300 μl of PBS. Twenty μl of chilled test solution was gently vortexed, added to a retainer and spun at 850 rpm for 3 minutes. The slides with deposited cells were removed and allowed to air dry. Finally, after the slides were completely dry, one drop of cyanoacrylate adhesive (Adhesive cyⅎ..ⅉacrylate, MIL-A-46050C, Type II, CL. 2 Three Bond of An ːrica) was placed over the concentrated area of cells followed by placement of a cover slip. The adhesive was allowed to set completely at room temperature before being stored at -20°C in the dark.

As with the superoxide analyses three seperate donors supplied PMNs on three different days. Each of the 18 Nhp sera were run once each day with and without human serum containing complement. The sequence of testing the Nhp sera was systematically changed each day to control for any decrease in PMN activity over time.

All slides were coded to blind the examiner during data collection. Twenty fields on each slide were evaluated, using a Vanox T (Olympus)(Type A emersion oil) microscope equiped with fluorescence (U filter) under 1000x magnification, tabulating the following information:

(a) the number of PMNs present;

(b) the number of PMNs that had either *P. gingivalis* in contact or inside the periphery of the cell;

(c) the number of *P. gingivalis* in contact with the periphery of the PMN; and

(d) the number of *P. gingivalis* that were inside the periphery of the PMN.

2.   Kinetics and killing assay

In order to assess the ability of PMNs to kill *P. gingivalis* or *P. gingivalis* to kill PMNs over time the same methodology as above was utilized with the following modifications. Human serum as a source of complement was added to all test solutions containing Nhp serum and all mixtures were incubated for 60 minutes in a 37°C water bath. Twenty µl samples were removed after gentle vortexing at time zero, 15, 30 and 60 minutes processed with the cytospin centrifuge. For this study only 3 Nhp sera were tested one from each category of antibody (i.e. natural, ligature, immunized), with PMNs from one donor. The data gathered from the slides included the same information as described above plus the number of PMN nuclei and *P. gingivalis* that were stained with propidium iodide as a marker of non-vital cells (Appendix C).

I.   Statistical Analyses

Statistical analysis was performed on all data collected except the kinetics portion of the ingestion, attachment and killing assay. Statistical tests were carried out using a microcomputer program, CSS:Statistica, (StatSoft) on an IBM PC. Differences between groups based on antibody induction methods were evaluated using a 2 Sample t-Test for the direct killing, superoxide anion and ingestion/attachment data. Antibody levels in ELISA units and superoxide release data were subjected to Spearman rank correlation analysis.

III. RESULTS

A. IgG Immunoglobulin Levels

Three subsets of serum (natural, ligature, and immunized) were drawn from twenty adult female cynomolgus monkeys. Natural sera (baseline) were drawn prior to any experimentation; ligature sera were drawn 6 to 10 weeks following ligation of three posterior teeth inducing periodontitis; and the immunized sera were collected after three immunizations of 10^9 formalin killed *P. gingivalis* and ligation induced periodontitis. The immunized sera were also drawn 6 to 10 weeks post ligature placement. Immunoglobulin titers were assessed by ELISA and recorded in ELISA units resulting in the immunized group producing significantly more IgG than the natural or ligature induced groups (Table 6).

B. Direct Killing Assay

The ability of Nhp serum to kill *P. gingivalis* was tested by using fresh cultures of *P. gingivalis* 3079.03 in combination with a 1:100 dilution of Nhp serum with or without complement. In initial experimental trials *P. gingivalis* was opsonized with Nhp serum for 30 minutes, followed by a 30 minute incubation period with complement. This resulted in little killing activity above control values. A second protocol was then developed which utilized a 30 minute incubation period with both the Nhp serum and complement being added simultaneously at the beginning of the assay. This procedure resulted in an increase in the amount of direct killing of *P. gingivalis*, which was especially evident in the immunized group sera. (Figure 10).

A mean and standard deviation was calculated for each serum group and a t-Test for two independent samples was performed comparing the groups. The serum

TABLE 6

IgG Anti-*P. gingivalis* Antibody in Nonhuman Primate Sera

Group	n=	MEAN	MEDIAN	STD DEV	MIN/MAX	p<
Natural	6	11.38[a]	9.995	8.29	3.02/23.84	----
Immunized	6	556.813	457.6	388.121	234/1306	.005
Ligature	6	22.83	15	22.619	3.38/60.9	----

[a] Data expressed in ELISA Units.

FIGURE 10 Direct Killing of *P. gingivalis* by Nhp Sera Plus Complement Using Two Incubation Protocols.

The amount of *P. gingivalis* killed directly by Nhp sera and complement is represented here with the percentage of *P. gingivalis* killed on the y axis and the amount of IgG expressed in ELISA units on the x-axis. The 30 minute incubation with serum plus 30 minute incubation with complement is represented with a solid line and shows less activity than the 30 minute incubation with both serum and complement together on the broken line. In all trials a 1:100 dilution of 5% bovine serum albumin was run as a control with a consistent direct kill rate of ~10%. This percentage was subtracted from the results represented here as background.

Direct Killing of P. gingivalis
by Nhp Sera Plus Complement
Using Two Incubation Protocols
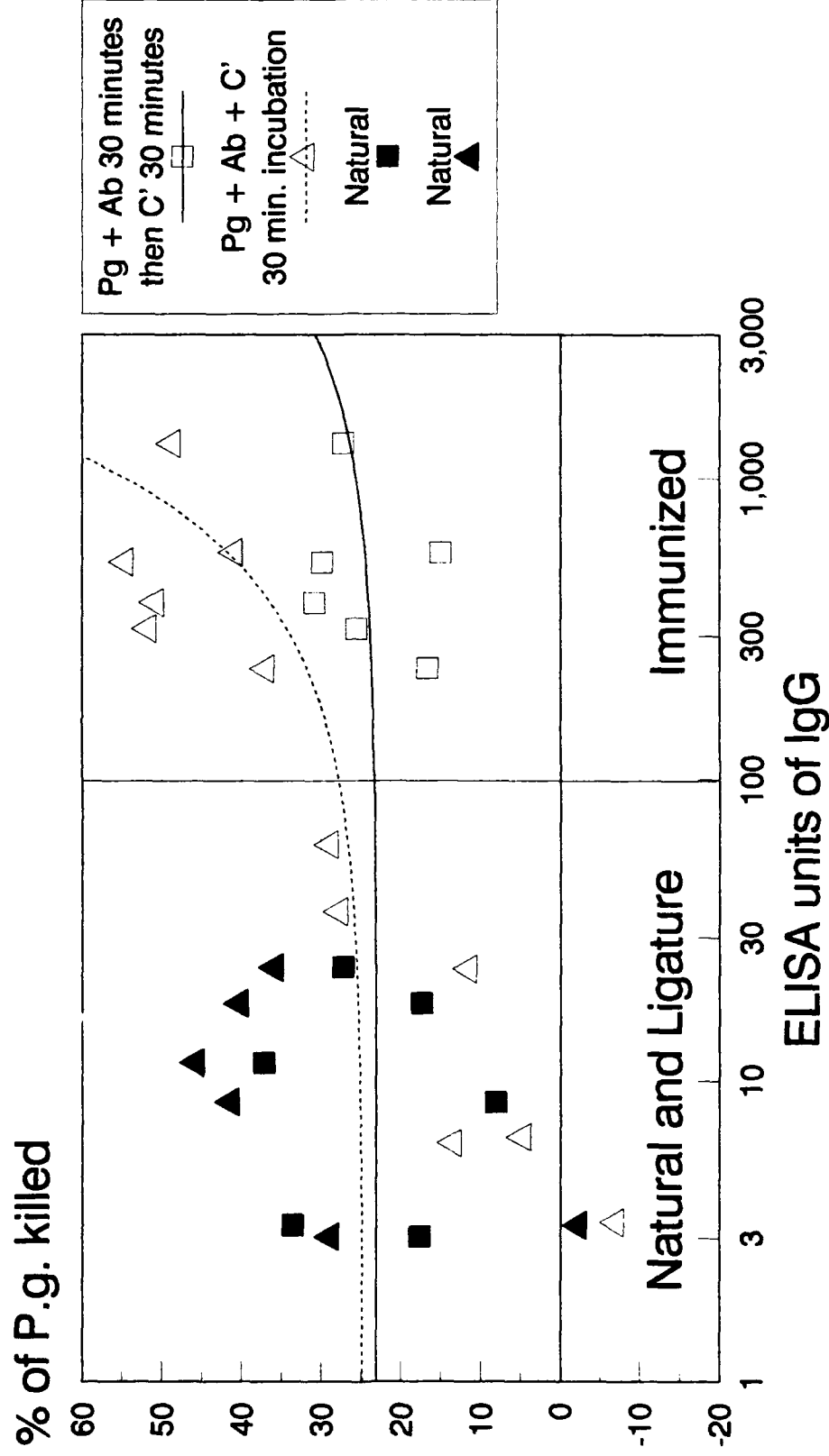
alone trials revealed an amount of killing in the absence of complement with no significant differences between the natural and immunized groups. The ligature group showed statistically lower levels of direct killing activity as compared to the natural and the immunized group sera (Figure 11).

When direct killing results were analyzed with Nhp serum plus complement, significantly more direct killing occurred in the immunized group compared to either the natural or the ligature group sera. There was no significant difference between the natural group and ligature group (Figure 11) in this assay system.

The results of the direct killing capacity of the Nhp serum plus complement trials were then compared to the amount of IgG antibody detected in each serum (Figure 12). A correlation of killing activity related to the titer of antibody was suggested but did not appear to be absolute. This inconsistency was most evident in the animals stimulated by immunization. In the sera containing lower titers of IgG, a difference in activity level was found, with some of the natural sera showing moderate to high levels of direct killing activity (8.5-18.13 ELISA units), and the majority of ligature sera, the least levels of activity.

C.   Superoxide Anion Assay

Freshly harvested human PMNs were reacted with *P. gingivalis* under two conditions: Nhp serum alone; and Nhp serum plus complement. The results were recorded in two forms: (i) the change in absorbance of the test solution resulting from a reduction in cytochrome C over time; and (ii) an estimate of the nanomoles of superoxide produced as determined from a net change in absorbance of the test solution. A mean and standard deviation were calculated for the amount of superoxide in each group and a comparison between groups was performed using a t-Test for two independent samples.

FIGURE 11 Direct Killing of *P. gingivalis* by Nhp Sera and Nhp Sera Plus Complement. The amount of *P. gingivalis* killed directly by a 30 minute incubation period with either Nhp serum or Nhp serum plus complement is shown with the percentage of *P. gingivalis* killed on the y-axis and the method of antibody induction represented on the x-axis. In the Nhp serum only assay, the immunized group killed significantly more *P. gingivalis* than the ligature group (* $p<0.03$) and the natural group killed significantly more *P. gingivalis* than the ligature group (* $p<0.008$). In the Nhp serum plus complement assay the immunized group killed significantly more *P. gingivalis* than either the ligature group (# $p<0.005$) or the natural group (# $p<0.04$). In all trials a 1:100 dilution of 5% bovine serum albumin was run as a control with a consistent direct kill rate of ~10%. This percentage was subtracted from the results represented here as background.

Direct Killing of P. gingivalis
by Nhp Sera and
Nhp Sera Plus Complement
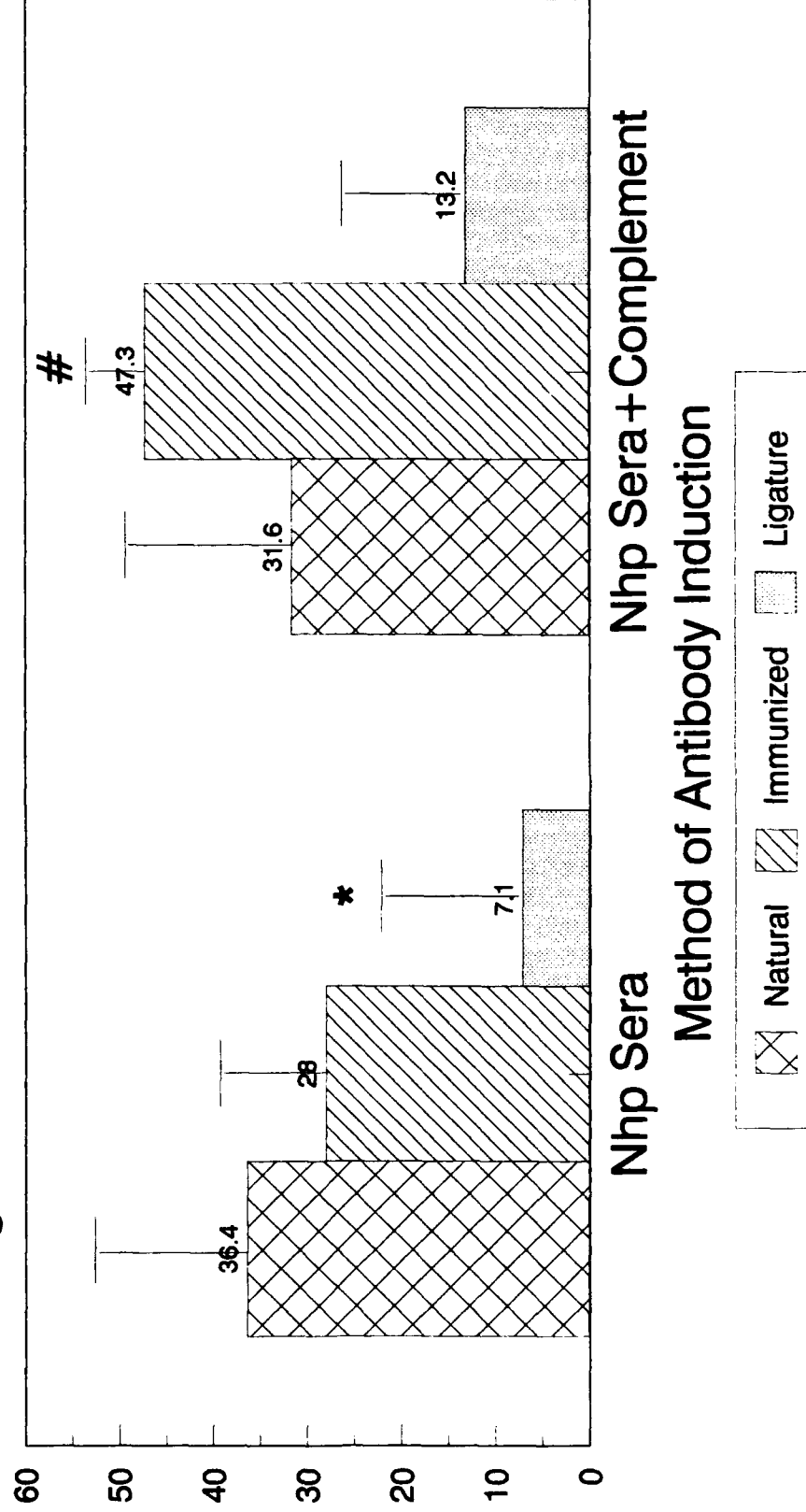
FIGURE 12 Percentage of Direct Killing Associated with Individual Nhp Sera.
All trials represented here were run in the presence of Nhp sera and complement. The percentage of *P. gingivalis* killed is shown on the y-axis and the IgG titer of anti-*P. gingivalis* immunoglobulin is represented for each of the 18 animals on the x-axis. (N=Natural group, i=Immunized group, L=Ligature group). In all trials a 1:100 dilution of 5% bovine serum albumin was run as a control with a consistent direct kill rate of around 10%. This percentage was subtracted from the results represented here as background.

Titer of IgG to P. gingivalis Compared to
Percentage of Direct Killing of P. gingivalis

Percentage of P gingivalis killed

% Direct Kill

ELISA UNITS OF IgG
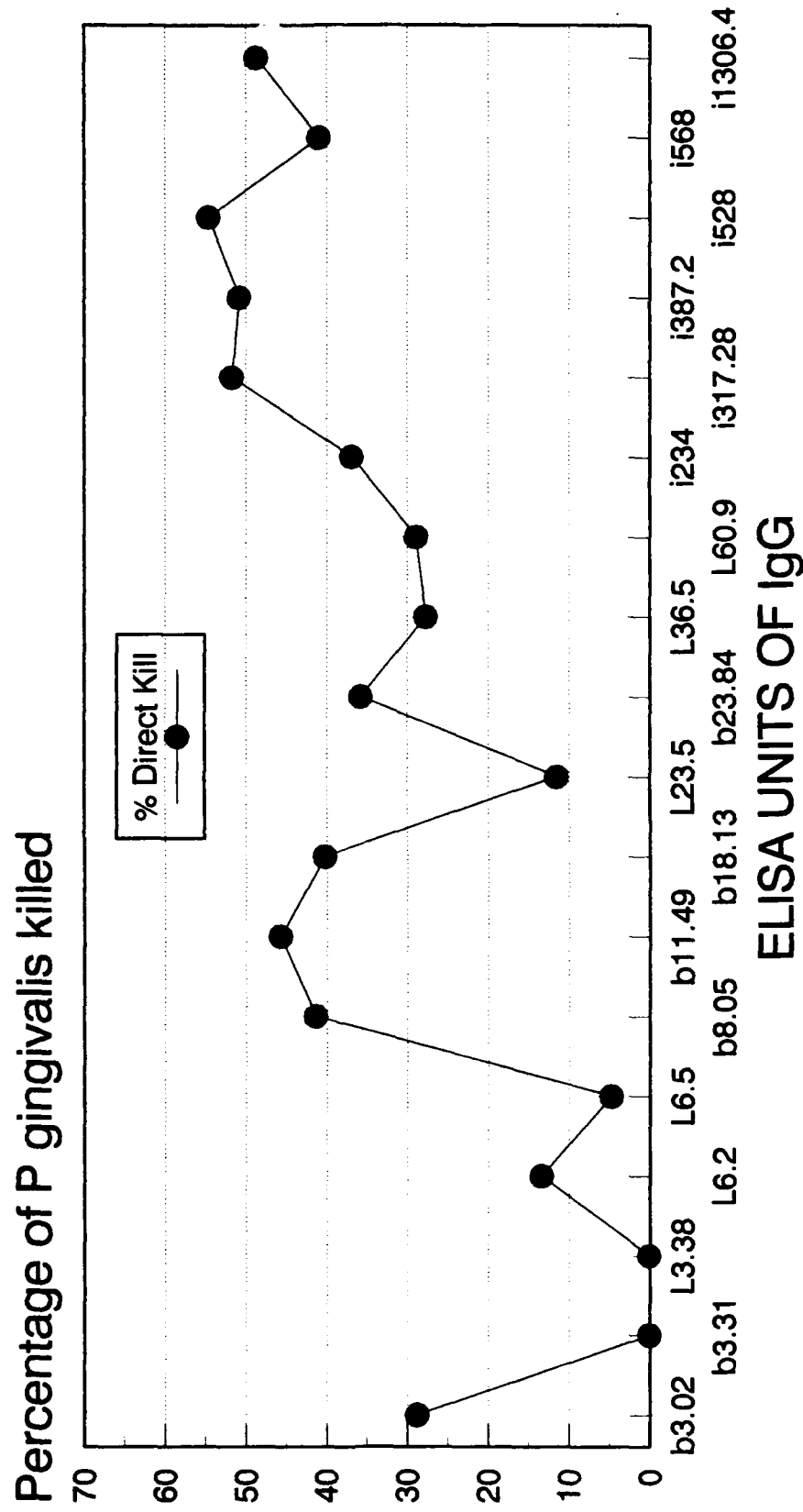
Percentage of Pg killed in assay with human C'.

b=Baseline, L=Ligature, i=Immunization

The change in absorbance over a 30 minute time period was recorded by a microcomputer and was converted to graph form. The following figures represent the mean changes for three trials conducted on three different days with separate PMN sources. The data showed that in the natural sera alone (Figure 13) three sera (#8, #10, #11) responded with a greater change in absorbance than the other three sera. The immunized sera (Figure 14) showed four examples (#1, #2, #3, #5) producing large changes in absorbance. The ligature sera (Figure 15) exhibited only 2 sera (#13, #17) which showed a change in absorbance over time.

The reactivity of the natural sera plus complement is graphically represented in figure 16. Only one serum (#11) appeared to enhance superoxide productivity by the PMNs. The immunized sera plus complement experiments (Figure 17) showed that all sera induced substantial changes in absorbance with only one serum (#6) at the low end of the scale. The ligature sera plus complement data (Figure 18) showed that only two sera (#13, #17) produced a substantial change in absorbance.

The control specimens in these assays included: (i) PMNs plus *P. gingivalis*; (ii) PMNs plus serum; (iii) PMNs plus complement; (iv) PMNs plus *P. gingivalis* plus complement; and (v) PMNs plus Nhp serum plus complement. They all showed minimal changes in absorbance over time (Figure 19). The PMN plus *P. gingivalis* sample represents 10 trials averaged together from three different donors of PMNs. The remaining controls were run during initial experimental trials and were found to produce little to no change in absorbance over a 15 minute time period.

When the kinetic absorbance data of superoxide production were meaned (Figure 20) for assay testing serum alone, the immunized group had the greatest change in absorbance followed by the natural group sera; the ligature group sera, and the control conditions demonstrated the least changes in absorbance. Meaned data of the serum plus complement trials (Figure 21) demonstrated that only the immunized

FIGURE 13 Superoxide Anion Released Over Time From Human Neutrophils in the Presence of Natural Serum and *P. gingivalis*. This graph represents the 6 natural sera reacted with *P. gingivalis* for 30 minutes. The absorbance is represented on the y-axis, and the time in seconds is represented on the x-axis. The graphed results for each sera represent the mean of three trials, run on three different days, with 3 different human donors (neutrophils). All absorbance levels were adjusted to begin at zero absorbance.

Superoxide Anion Released Over Time From Human Neutrophils in the Presence of Natural Serum and P. gingivalis

Absorbance values adjusted to all begin at zero.
Three trials averaged for each serum.

FIGURE 14 Superoxide Anion Released Over Time From Human Neutrophils in the Presence of Immunized Serum and *P. gingivalis*. This graph represents the 6 immunized sera reacted with *P. gingivalis* for 30 minutes. The absorbance is represented on the y-axis, and the time in seconds is represented on the x-axis. The graphed results for each sera represent the mean of three trials, run on three different days, with 3 different human donors (neutrophils). All absorbance levels were adjusted to begin at zero absorbance.

Superoxide Anion Released Over Time From Human Neutrophils in the Presence of Immunized Serum and P. gingivalis

Serum #1   Serum #2   Serum #3   Serum #4   Serum #5   Serum #6

Absorbance values adjusted to all begin at zero.
Three trials averaged for each serum.

FIGURE 15 Superoxide Anion Released Over Time From Human Neutrophils in the Presence of Ligature Serum and *P. gingivalis.* This graph represents the 6 ligature sera reacted with *P. gingivalis* for 30 minutes. The absorbance is represented on the y-axis, and the time in seconds is represented on the x-axis. The graphed results for each sera represent the mean of three trials, run on three different days, with 3 different human donors (neutrophils). All absorbance levels were adjusted to begin at zero absorbance.

Superoxide Anion Released Over Time From Human Neutrophils in the Presence of Ligature Serum and P. gingivalis

Absorbance values adjusted to all begin at zero.
Three trials averaged for each serum.

FIGURE 16 Superoxide Anion Released Over Time From Human Neutrophils in the Presence of Natural Serum, Complement and *P. gingivalis.* This graph represents the 6 natural sera and complement reacted with *P. gingivalis* for 30 minutes. The absorbance is represented on the y-axis, and the time in seconds is represented on the x-axis. The graphed results for each sera represent the mean of three trials, run on three different days, with 3 different human donors (neutrophils). All absorbance levels were adjusted to begin at zero absorbance.

Superoxide Anion Released Over Time From Human Neutrophils in the Presence of Natural Serum, Complement and P. gingivalis
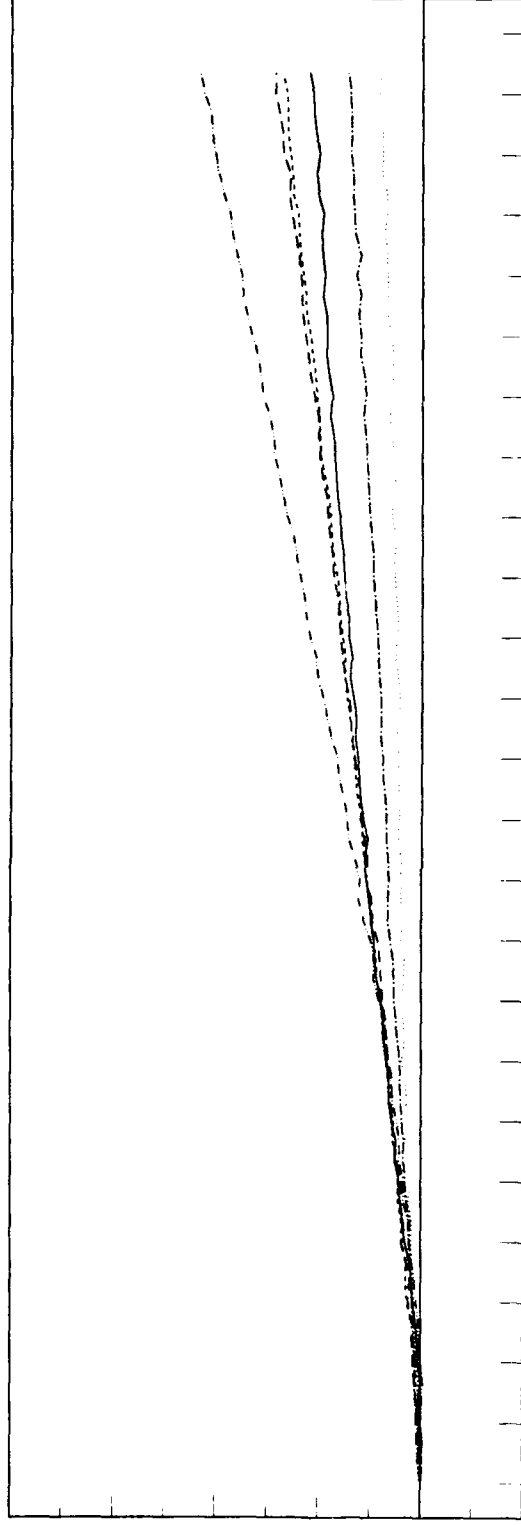
ABS

Time in Seconds

Serum #7 ——— Serum #8 ·········· Serum #9 – – – – Serum #10 – – – – Serum #11 – · – · – Serum #12 – ·· – ··

Absorbance values adjusted to all begin at zero.
Three trials averaged for each serum.

FIGURE 17 Superoxide Anion Released Over Time From Human Neutrophils in the Presence of Immunized Serum, Complement and *P. gingivalis*. This graph represents the 6 immunized sera and complement reacted with *P. gingivalis* for 30 minutes. The absorbance is represented on the y-axis, and the time in seconds is represented on the x-axis. The graphed results for each sera represent the mean of three trials, run on three different days, with 3 different human donors (neutrophils). All absorbance levels were adjusted to begin at zero absorbance.

Superoxide Anion Released Over Time From Human Neutrophils in the Presence of Immunized Serum, Complement and P. gingivalis

Absorbance values adjusted to all begin at zero.
Three trials averaged for each serum.

FIGURE 18 Superoxide Anion Released Over Time From Human Neutrophils in the Presence of Ligature Serum, Complement and *P. gingivalis.* This graph represents the 6 ligature sera and complement reacted with *P. gingivalis* for 30 minutes. The absorbance is represented on the y-axis, and the time in seconds is represented on the x-axis. The graphed results for each sera represent the mean of three trials, run on three different days, with 3 different human donors (neutrophils). All absorbance levels were adjusted to begin at zero absorbance.

Superoxide Anion Released Over Time From Human Neutrophils in the Presence of Ligature Serum, Complement and P. gingivalis
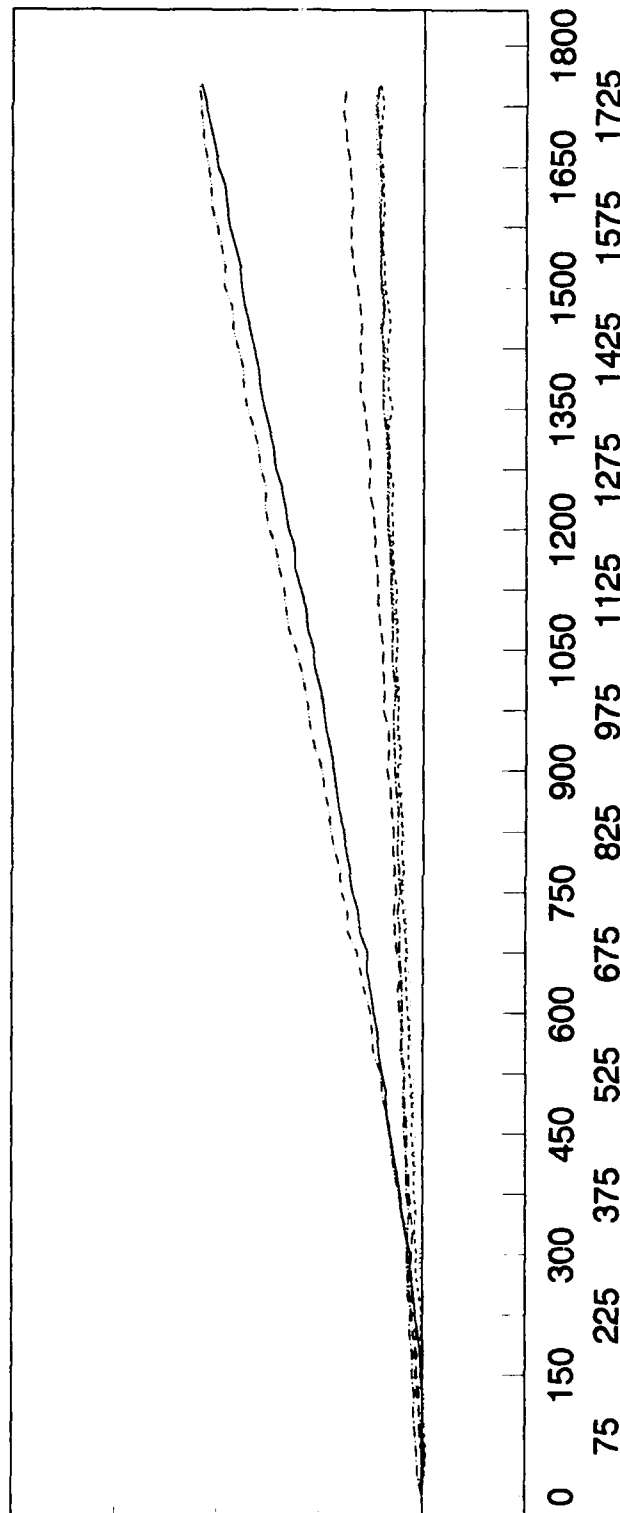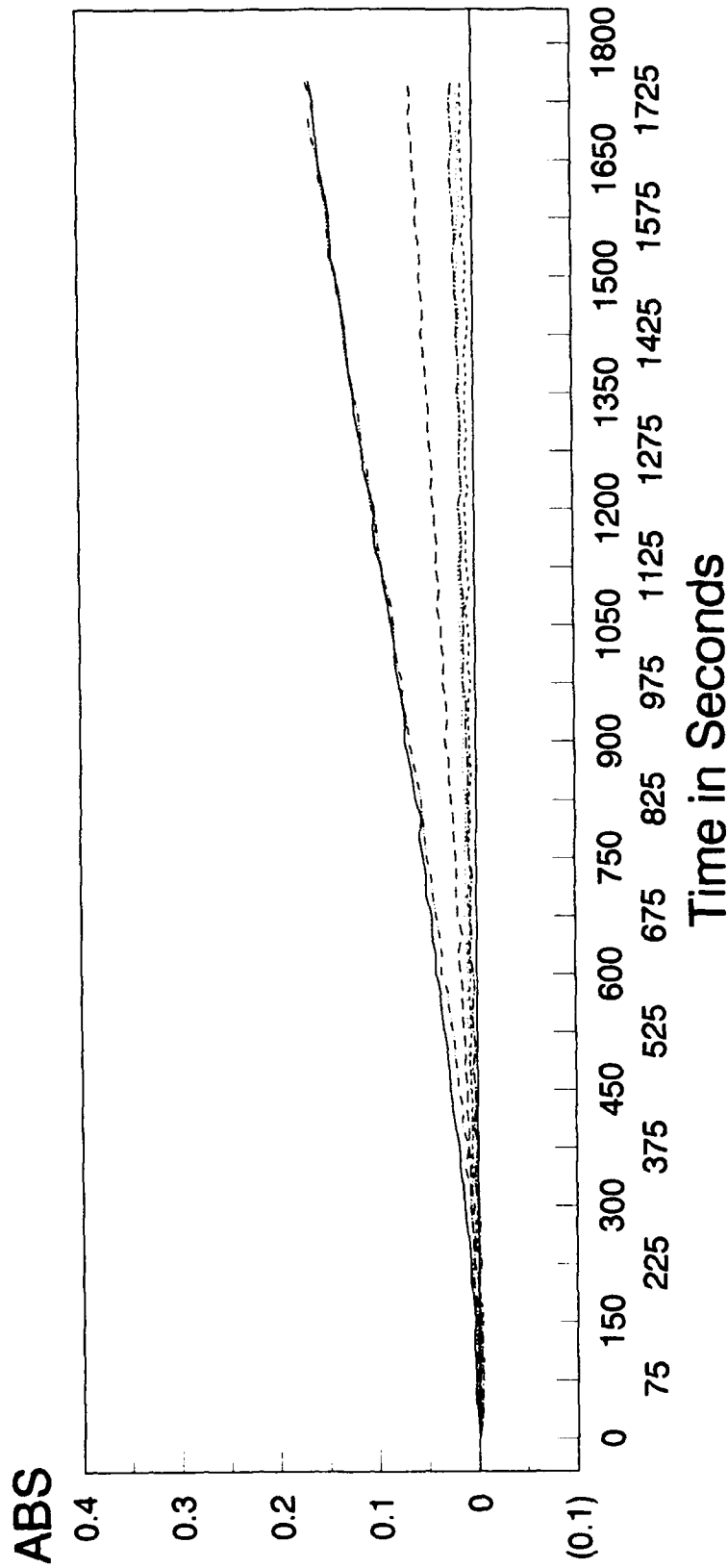
Serum #13   Serum #14   Serum #15   Serum #16   Serum #17   Serum #18

Absorbance values adjusted to all begin at zero.
Three trials averaged for each serum.

FIGURE 19 Superoxide Anion Released Over Time From Human Neutrophils Control Trials. This graph represents the 5 control groups. The PMN + Pg (*P. gingivalis*) group is represented by the mean of 10 trials. The remaining groups are shown as 15 minute incubation periods and show minimal change in absorbance resulting in no release of superoxide. All absorbance levels were adjusted to begin at zero absorbance.

Superoxide Anion Released Over Time From Human Neutrophils Control Trials

ABS

Time in Seconds
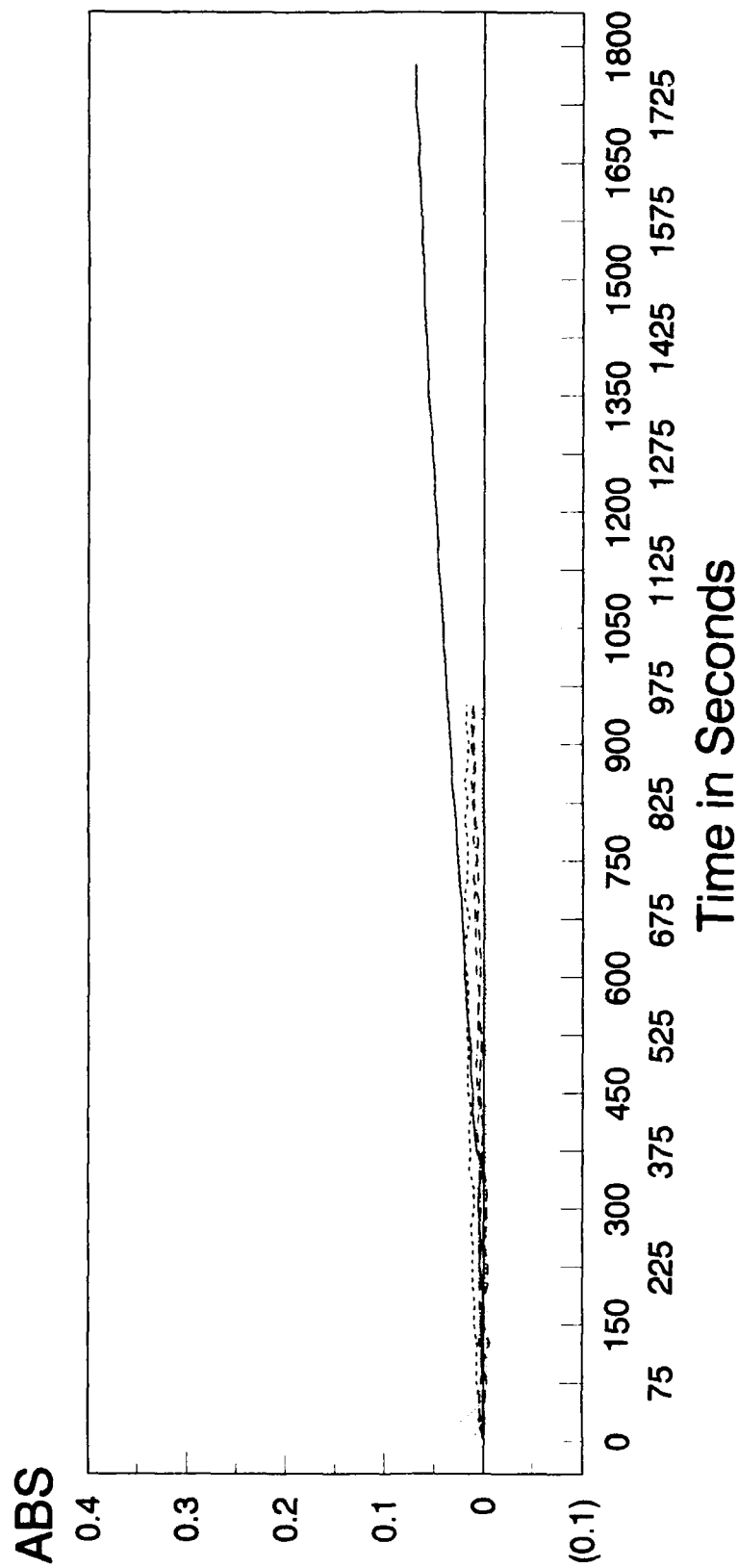
PMN+Pg   PMN+Serum   PMN+C'   PMN+Pg+C'   PMN+Serum+C'

Absorbance values adjusted to all begin at zero.

FIGURE 20 Superoxide Anion Released Over Time From Human Neutrophils Means for Each Serum Group Compared. This graph represents the change in absorbance on the y-axis over 30 minutes for PMN + Pg (Control), immunized, natural, and ligature sera. These values were meaned for 18 trials for each sera and 10 trials for the control results. All absorbance levels were adjusted to begin at zero absorbance.

Superoxide Anion Released Over Time From Human Neutrophils

Means for Each Serum Group Compared

ABS

0.4
0.3
0.2
0.1
0
(0.1)

0 75 150 225 300 375 450 525 600 675 750 825 900 975 1050 1125 1200 1275 1350 1425 1500 1575 1650 1725 1800

Time in Seconds

PMN+Pg   Immunized   Baseline   Ligature
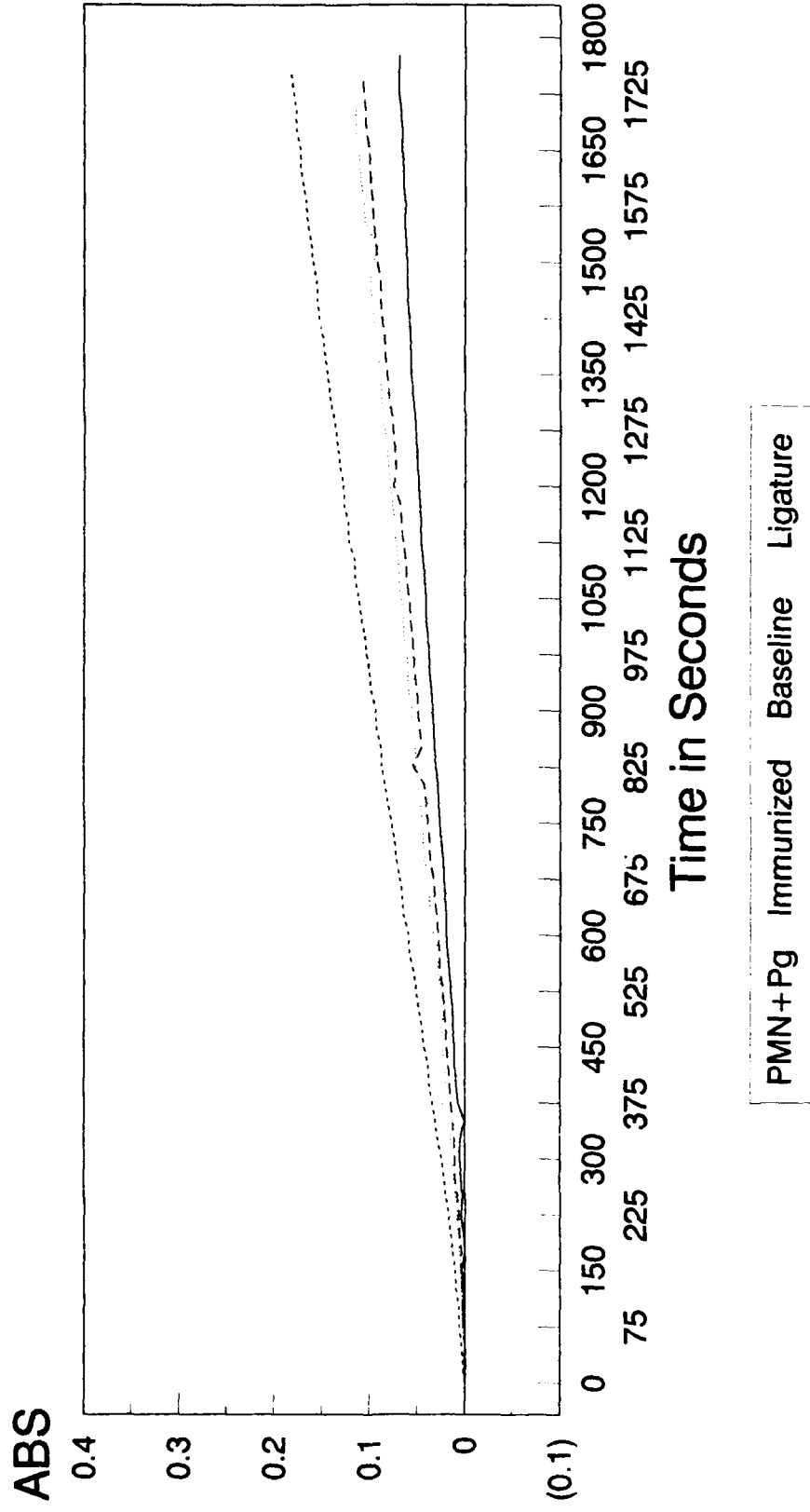
Absorbance values adjusted to all begin at zero.
Three trials averaged for each serum.

FIGURE 21 Superoxide Anion Released Over Time From Human Neutrophils Means for Each Serum Group Plus C' Compared. This graph represents the change in absorbance on the y-axis over 30 minutes for PMN + Pg (Control) immunized, natural, and ligature sera plus complement. These values were meaned for 18 trials for each sera and 4 trials for the control results. All absorbance levels were adjusted to begin at zero absorbance.

Superoxide Anion Released Over Time
From Human Neutrophils
Means for Each Serum Group Plus C' Compared
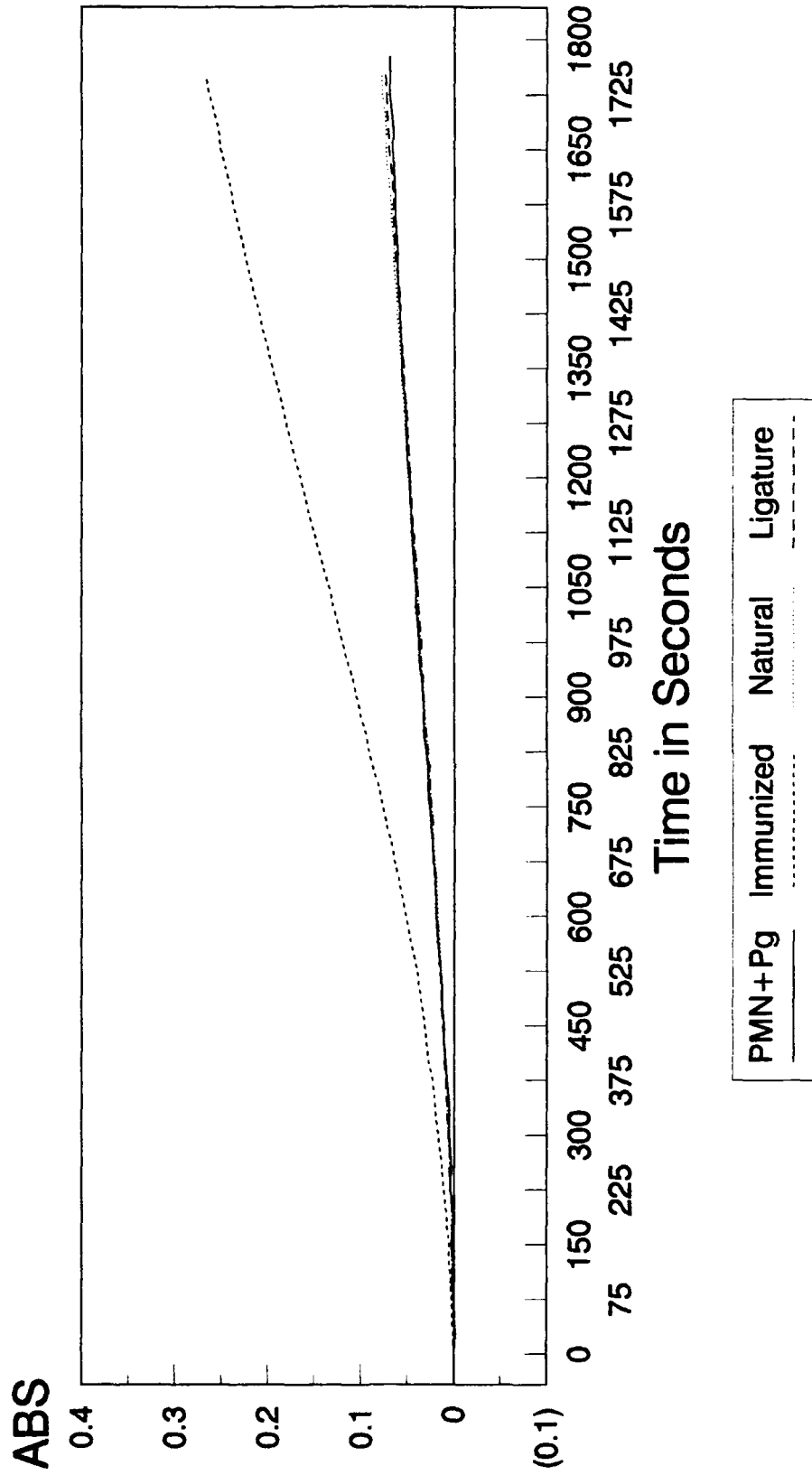
Absorbance values adjusted to all begin at zero.
Three trials averaged for each serum.

group showed a substantial change in absorbance, with little difference among the other groups.

Statistical analysis of the serum alone studies revealed that the meaned nanomolar production of superoxide from each group and each experimental condition resulted in (Figure 22) no statistical differences among the sera, or between the serum groups and the control. In contrast, when immunized group sera plus complement was tested, significantly more superoxide was produced than the natural group ($p < 0.008$), the ligature group ($p < 0.04$) or the control assays ($p < 0.02$).

This data suggested that the immunized sera reacting with *P. gingivalis* induced significantly more superoxide anion release than any of the other serotypes, and that complement was a key component leading to maximum release of superoxide anion from the PMNs. The data describing change in absorbance over time suggested substantial differences among sera within the same group in the ability to bind to *P. gingivalis* and interact with human PMNs.

D. Ingestion and Attachment Assay

Freshly harvested human PMNs were incubated with *P. gingivalis*, labeled with fluorescent dyes (DAPI and propidium iodide), for 15 minutes in combination with: (i) Nhp serum alone; or (ii) Nhp serum plus complement. The reaction mixture was then placed onto a slide by cytocentrifugation and preserved for future enumeration under fluorescent microscopy. The variables recorded for this assay included the total number of PMNs present, the number of active PMNs (i.e. *P. gingivalis* either attached or ingested) (Figure 23), the number of *P. gingivalis* attached to the periphery of the PMN and the number ingested by the PMN. Initially, the number of non-vital PMNs and

FIGURE 22 Neutrophil Superoxide Assay. This graph presents the results of the amount of superoxide released in nMoles on the y-axis and the conditions of the assay on the x-axis. The left cluster shows the mean amount of superoxide released from Natural, Immunized, Ligature sera and Control. There are no significant differences between these 4 groups. The right cluster shows the mean amount of superoxide released from the same four groups in the presence of serum and complement. Signficantly less superoxide was produced by the natural group ($*p<0.008$), ligature group ($\#p<0.04$) and the control group ($\$p<0.02$) as compared to the immunized group. There was no statistical difference between the natural, ligature or control groups. T represents the standard deviation for each group.

Neutrophil Superoxide Assay

nMoles of Superoxide

Method of Antibody Induction

Natural Immunized Ligature Control
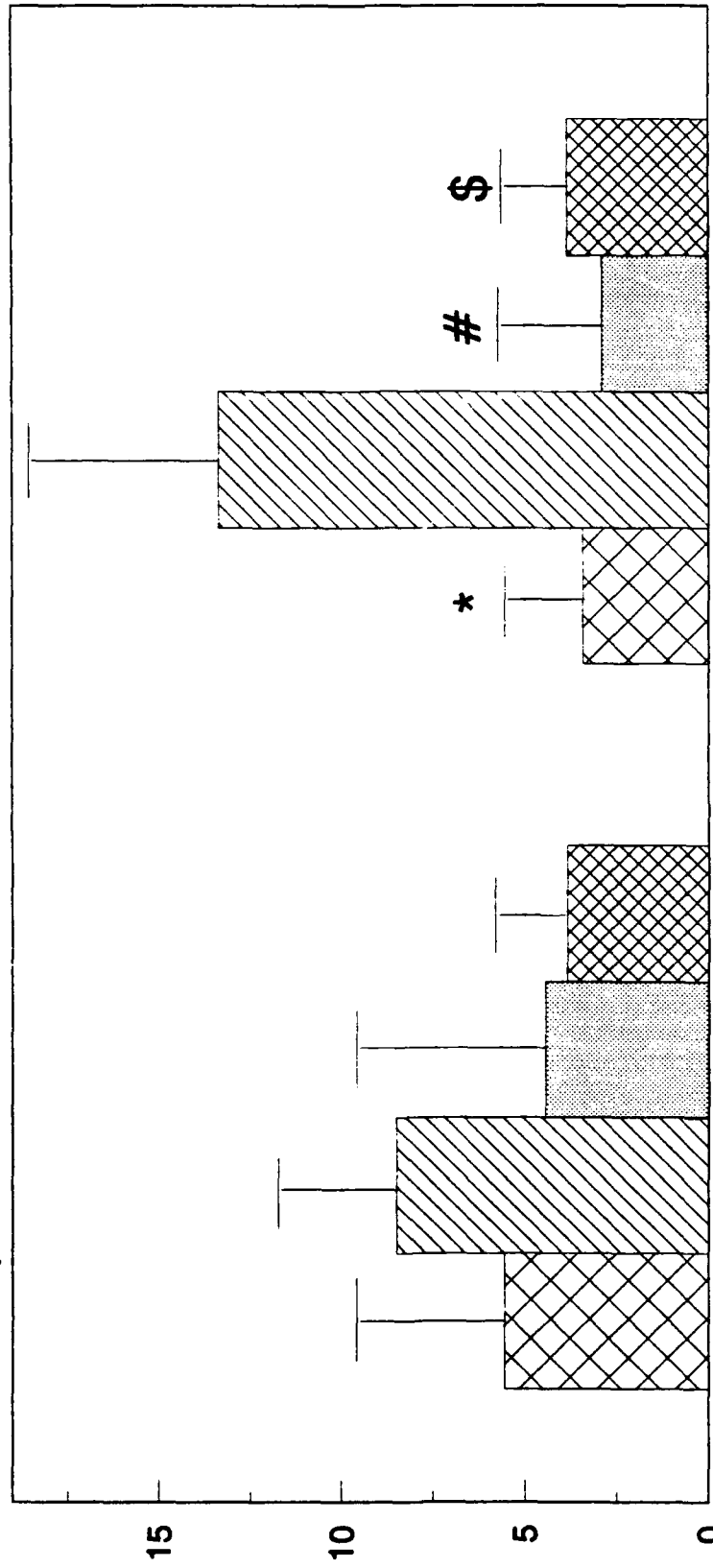
Significance:
* (p<0.008) # (p<0.04) $ (p<0.02)
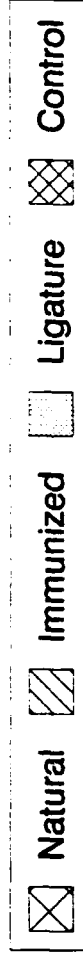
FIGURE 23 Attachment/Ingestion Assay Under Fluorescent Microscope

A=Active PMN, I=Inactive PMN, PA=P. gingivalis attached to PMN periphery, PI=P. gingivalis ingested by PMN.
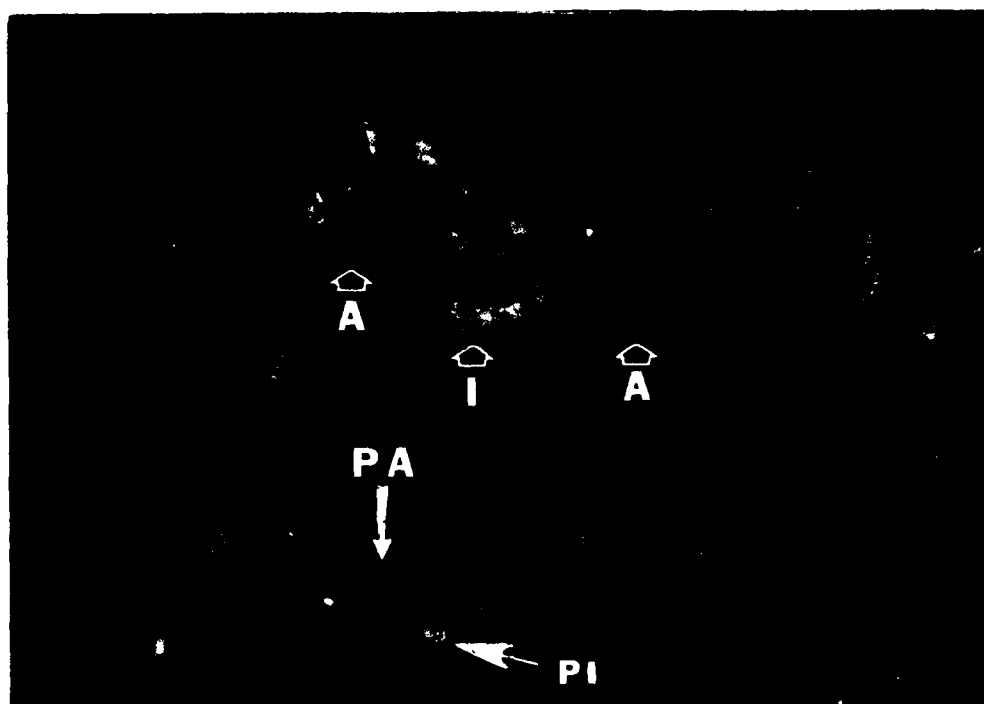
non-vital *P. gingivalis* present were considered as an outcome variable. However, so few non-vital cells were found in the 15 minute incubation period that documentation of these variables was discontinued.

The initial assay was to determine if the presence of different serum or serum plus complement conditions would alter the percentage of PMNs participating in the reaction. No significant differences were found within each of the clusters for the number of PMNs that were active in association with each serum type. It appears that 65-75% of the PMNs participated in the reaction in the absence of NEM and approximately 45-50% participated in the NEM treated assays (Figure 24).

The influence of complement on the reaction between the PMNs and *P. gingivalis*, as related to the total number of *P. gingivalis* attached and ingested per active PMN, (Figure 25) revealed that this interaction appeared to be complement independent. There were no significant differences among any of the types of serum when tested with serum alone or with serum plus complement.

An additional outcome variable was to determine differences in the number of *P. gingivalis* attached to the periphery of the active PMNs between control (PMNs plus *P. gingivalis*) and serum treatment conditions. It was found that (Figure 26) irrespective of the serum conditions and the presence of complement, no significant differences existed among any of the groups. Thus, it appeared that the interaction between the PMN and *P. gingivalis* leading to attachment was indepedent of the presence of serum or complement.

A final outcome to be examined was the interaction between the PMNs and *P. gingivalis* that resulted in *P. gingivalis* being ingested by active PMNs. The data revealed that the immunized sera significantly enhanced *P. gingivalis* ingestion per active PMN (p<0.005) when compared to any of the other groups (Figure 27). This

FIGURE 24 Percentage of Active PMNs (with *P. gingivalis* Attached or Ingested). This graph represents the percentage of PMNs that had *P. gingivalis* either attached or ingested as observed under fluorescent microscopy for the immunized, natural and ligature sera alone, with complement and with NEM. The conditions are listed on the y-axis. The percentage of PMNs reacting is shown on the x-axis. No significant differences were found within each cluster for PMN activity.

Percentage of Active PMNs
(with P. gingivalis Attached or Ingested)

Condition

Serum Alone

Serum + NEM

Serum + C'

Serum + C' + NEM

Percentage of PMNs Active

0   20   40   60   80   100

Immunized   Natural   Ligature
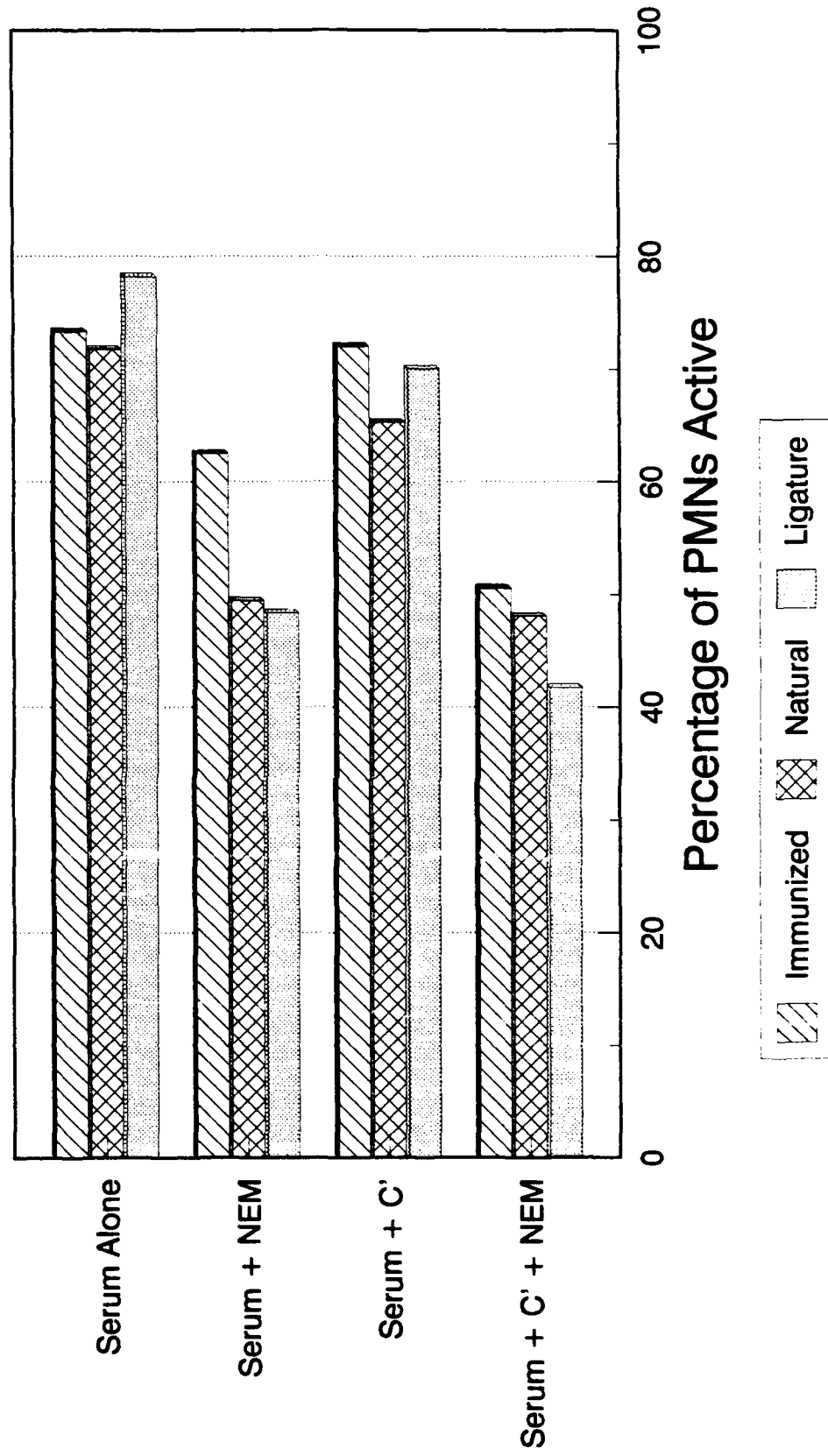
No Significant differences within clusters

FIGURE 25 Total *P. gingivalis* (attached plus ingested) per Active PMN Serum Alone versus Serum plus Complement. This graph compares the total number of *P. gingivalis* either attached or ingested per active PMN on the y-axis and the method of antibody induction on the x-axis. One bar in each cluster is with serum alone and the other is with serum plus complement. There were no significant differences between the bars in each cluster. T represents the standard deviation for each group.

Total P. gingivalis (attached plus ingested) per Active PMN

Serum Alone versus Serum plus Complement

No Significant Differences Within Each Cluster
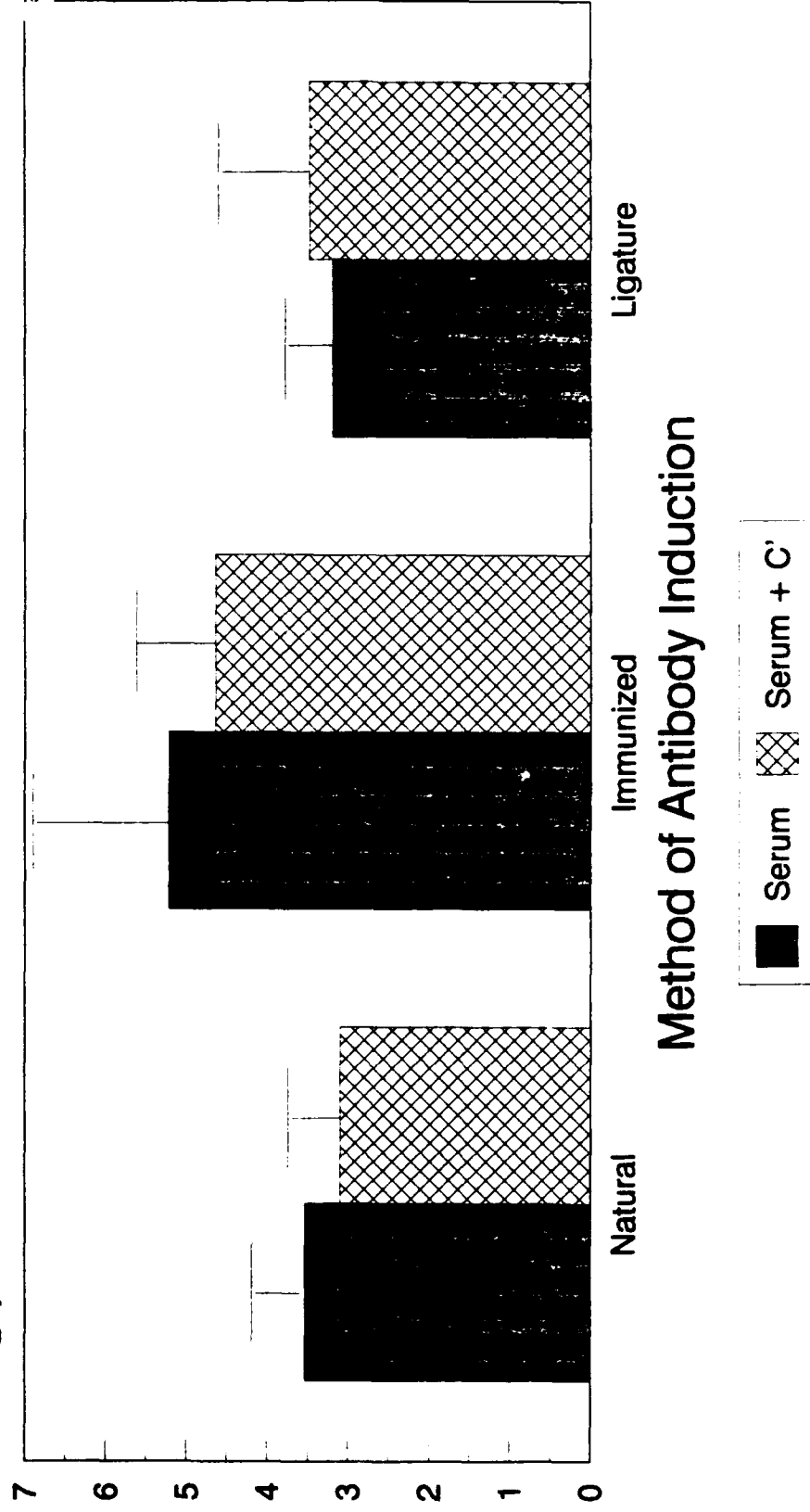
FIGURE 26 Attachment Assay *P. gingivalis* Attached per Active PMN. This graph, on the y-axis, presents the number *P. gingivalis* attached to the periphery of the *P. gingivalis* positive PMNs. The conditions of the assay are presented on the x-axis with the left cluster showing serum alone and the right cluster showing serum plus complement. Within each cluster no significant differences were found. T represents the standard deviation for each group.

Attachment Assay
P. gingivalis Attached per Active PMN

Pg Attach/Active PMN

Method of Antibody Induction

Natural   Immunized   Ligature   Control
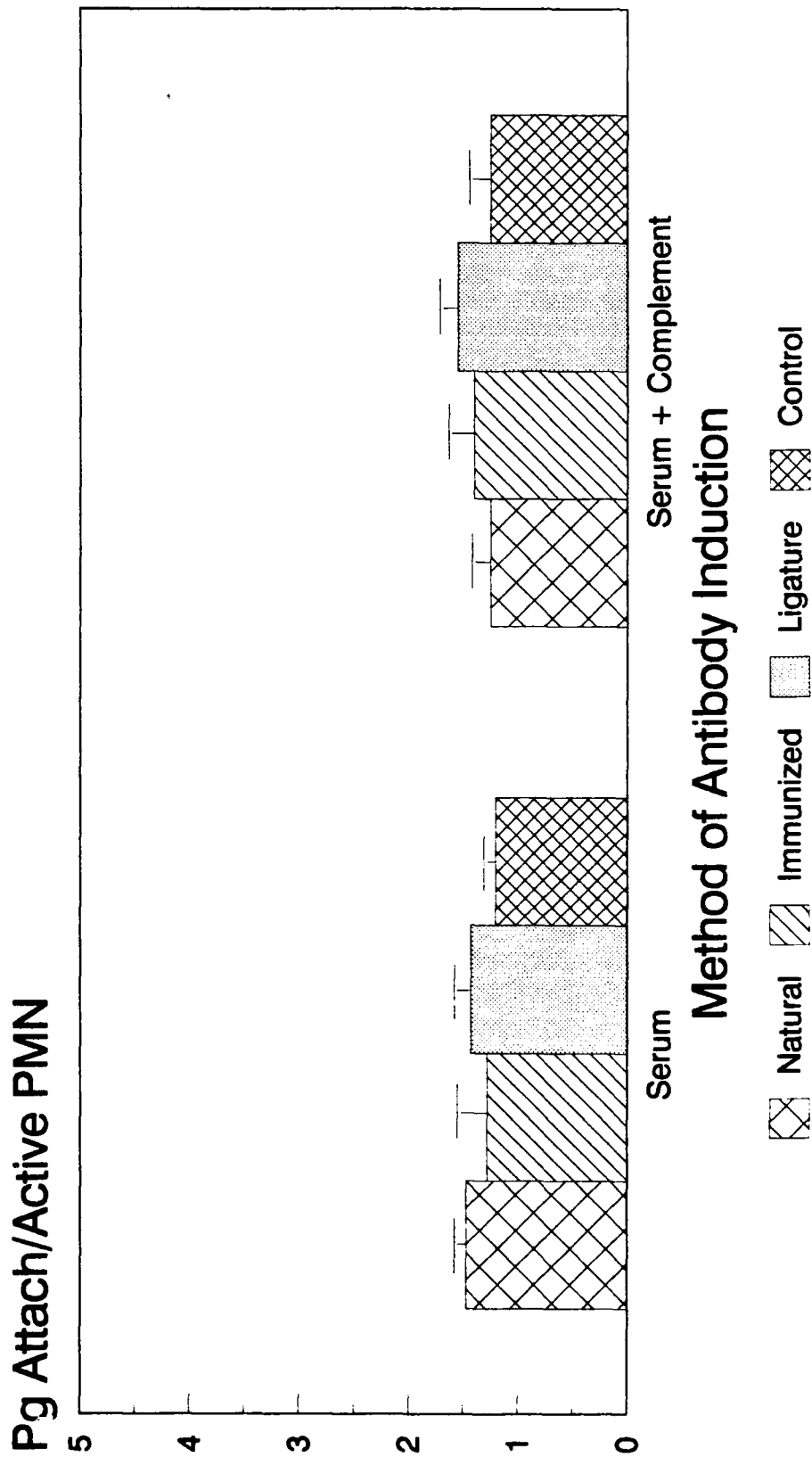
Significance:
No significant difference between any groups.

FIGURE 27 Ingestion Assay *P. gingivalis* Ingested per Active PMN. This graph, on the y-axis, presents the number of *P. gingivalis* ingested per *P. gingivalis* positive PMNs. The conditions of the assay are presented on the x-axis with the left cluster showing serum alone and the right cluster showing serum plus complement. The number of *P. gingivalis* ingested has been corrected to compensate for experimental error by subtracting the number of NEM control *P. gingivalis* that were found within the periphery of the PMNs. In the serum alone group the immunized group ingested significantly more *P. gingivalis* (*$p<0.005$) than the other three groups, with no differences found between the other three groups. In the serum plus complement group the immunized group ingested significantly more *P. gingivalis* (*$p<0.005$) than the other three groups, with no differences found between the other three groups. T represents the standard deviation for each group.

Ingestion Assay
P. gingivalis Ingested per Active PMN

Pg Ingested/Active PMN

Method of Antibody Induction

Serum Alone

Serum + Complement

Natural   Immunized   Ligature   Control
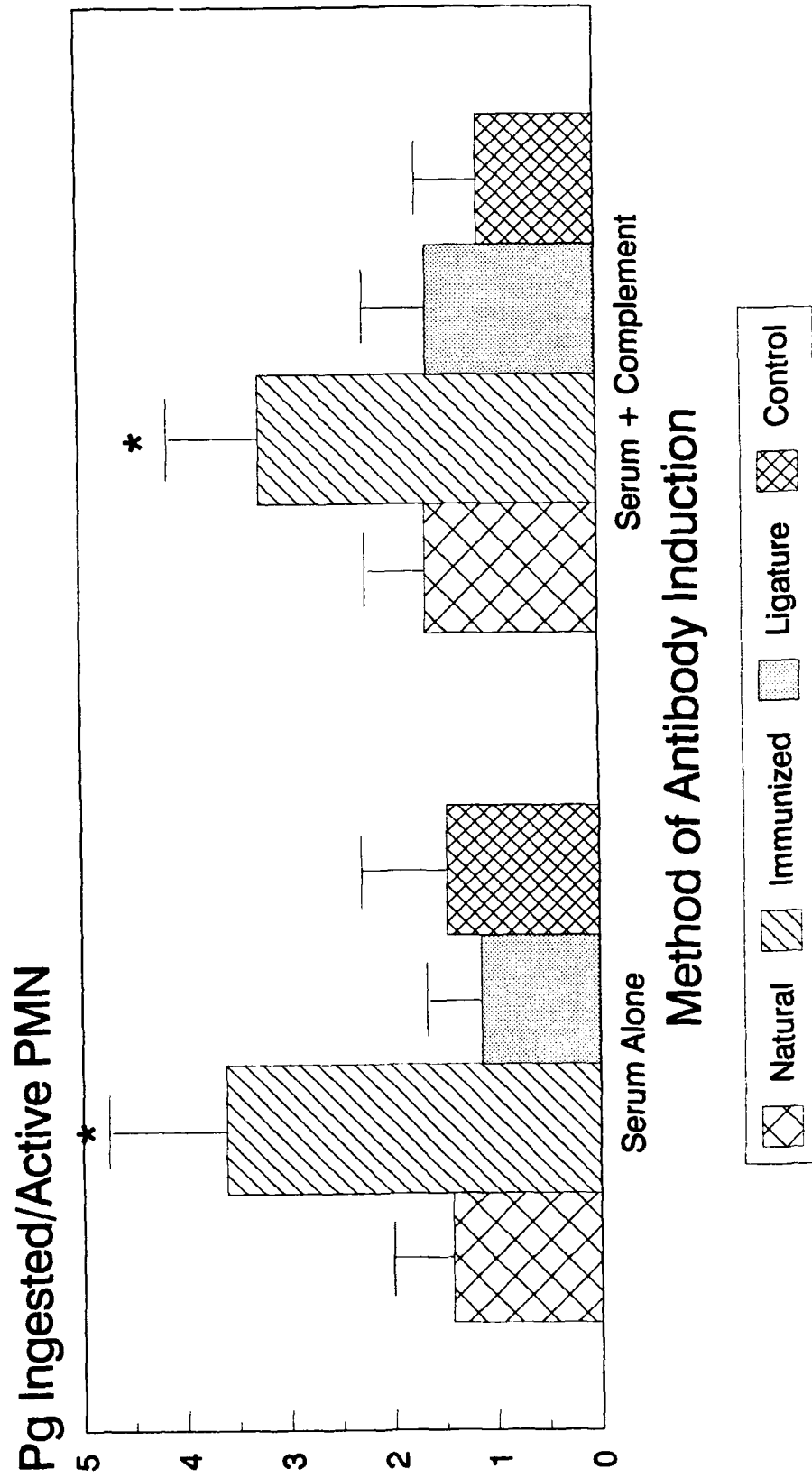
Significance:
* P<0.005

higher level of interaction appeared to be complement independent. Also, the number of *P. gingivalis* ingested was corrected to compensate for visual errors by subtracting the number of *P. gingivalis* that were found within the periphery of NEM treated PMNs (control) from each of the respective non-NEM treated test samples.

A summary of this data is shown in figures 28 and 29. The results indicated that comparison of the number of *P. gingivalis* attached, to the number ingested per active PMN (with and without complement) indicated that the number of attached bacteria remained fairly constant, but the number ingested varied depending upon the serum type being tested. When the total number of *P. gingivalis* both attached and ingested per active PMN was compared among serum types and conditions (Figure 30) the ligature group sera contributed less to this activity than the immunized sera under all conditions. The natural group sera was found to be less effective than the immunized group when serum plus complement was used and more reactive than the ligature group when serum or complement plus serum was tested. The ligature group sera was minimally effective and in most cases was comparable to the control values (*P. gingivalis* plus PMNs) in these tests.

As mentioned above, there existed the potential for visual error when assessing the location of individual bacteria. *P. gingivalis* appearing within the perimeter of the PMN may have been located in one of two places, actually within the PMN or attached to the top surface of the PMN. In the 2-dimensional view of a microscope an absolute delineation cannot be made. In order to adjust for this problem, an NEM control was included for each assay system. NEM physiologically shuts down the phagocytosing abilities of PMNs leaving the normal number of surface receptors exposed. Thus, the number of *P. gingivalis* observed within the periphery of the PMNs in the NEM conditions were subtracted from the homologous non-NEM tests to arrive at a corrected estimate of *P. gingivalis* ingested. The numbers of *P. gingivalis* that were observed

FIGURE 28 *P. gingivalis* Attached/Ingested per Active PMN Nonhuman Primate Serum Alone. This graph presents the number of *P. gingivalis* either attached or ingested in a stacked bar for each method of antibody induction when serum alone was used. The bottom half of the bar presents the percentage of the total *P. gingivalis* that was attached only, and the top of the bar presents the percentage of the total *P. gingivalis* that was ingested only. T represents the standard deviation for each group.

P. gingivalis Attached/Ingested per Active PMN

Nonhuman Primate Serum Alone

Number of P.g. per Active PMN (y-axis, scale 0 to 5)

Method of Antibody Induction (x-axis: Natural, Immunized, Ligature, Control)
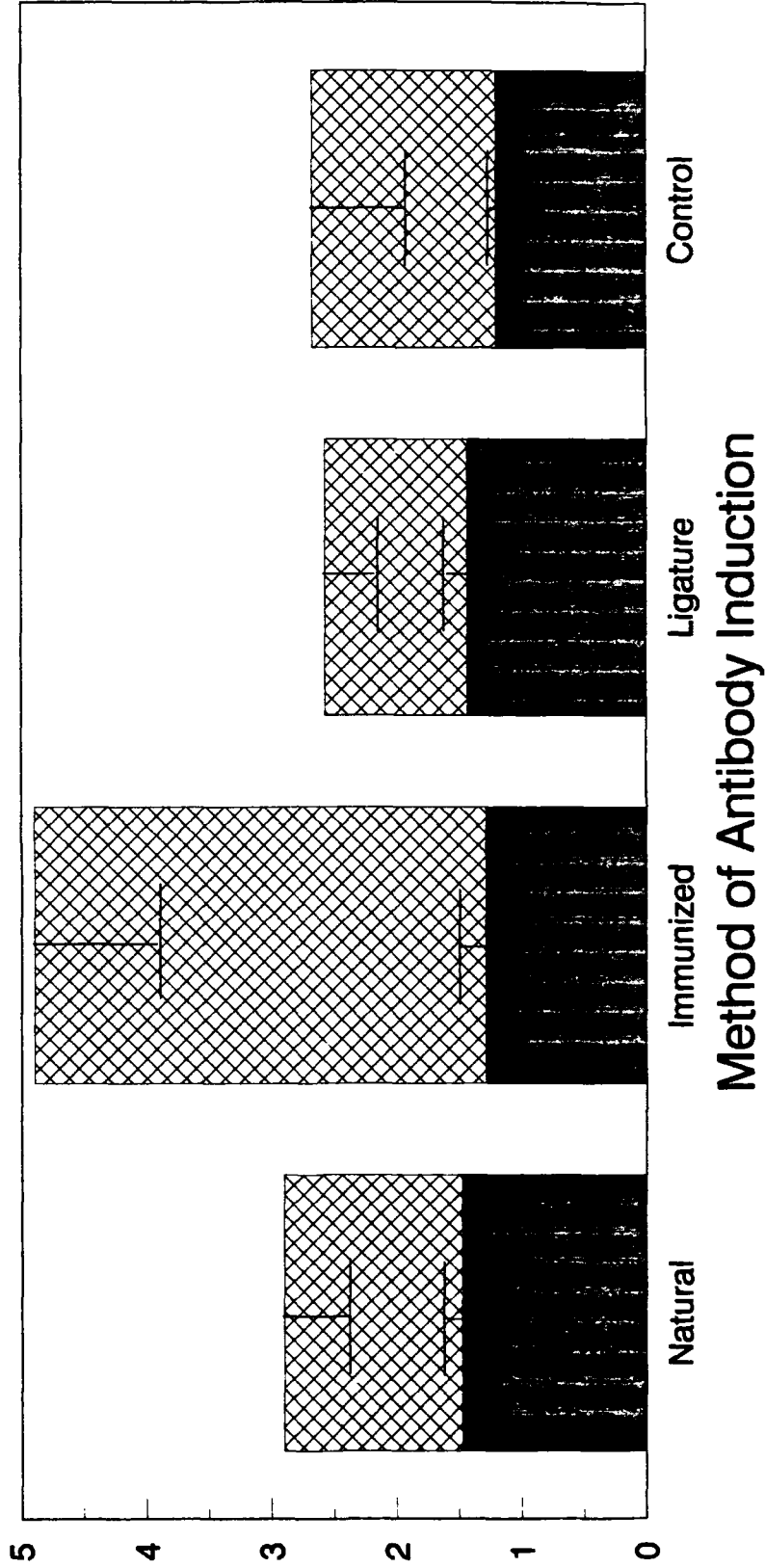
Legend: ■ % P.g. Attached ⬚ % P.g. Ingested

FIGURE 29 *P. gingivalis* Attached/Ingested per Active PMN Nonhuman Primate Serum plus Complement. This graph presents the number of *P. gingivalis* either attached or ingested in a stacked bar for each method of antibody induction when serum plus complement was used. The bottom half of the bar presents the percentage of the total *P. gingivalis* that was attached only, and the top of the bar presents the percentage of the total *P. gingivalis* that was ingested only. T represents the standard deviation for each group.

P. gingivalis Attached/Ingested per Active PMN
Nonhuman Primate Serum plus Complement

FIGURE 30 Total *P. gingivalis* (attached plus ingested) per Active PMN. This graph, on the y-axis, presents the total number of *P. gingivalis* per *P. gingivalis* positive PMN (Active). The conditions of the assay are presented on the x-axis. When serum alone was used the ligature group had significantly fewer *P. gingivalis* per active PMN ($p < 0.04$) than the immunized group. There was no difference found between the natural group and the other two groups. When serum plus NEM was used the ligature group had significantly fewer *P. gingivalis* per active PMN ($p < 0.003$) than the immunized group. There was no difference found between the natural group and the other two groups. When serum plus complement was used the ligature group ($p < 0.04$) and the natural group ($p < 0.009$) had significantly fewer *P. gingivalis* per active PMN than the immunized group. When serum and complement plus NEM was used the ligature group had significantly fewer *P. gingivalis* per active PMN than the natural group ($p < 0.0004$) and the immunized group ($p < 0.02$). No difference was found between the natural and the immunized groups under these conditions. T represents the standard deviation for each group.

Total P. gingivalis (attached plus ingested) per Active PMN
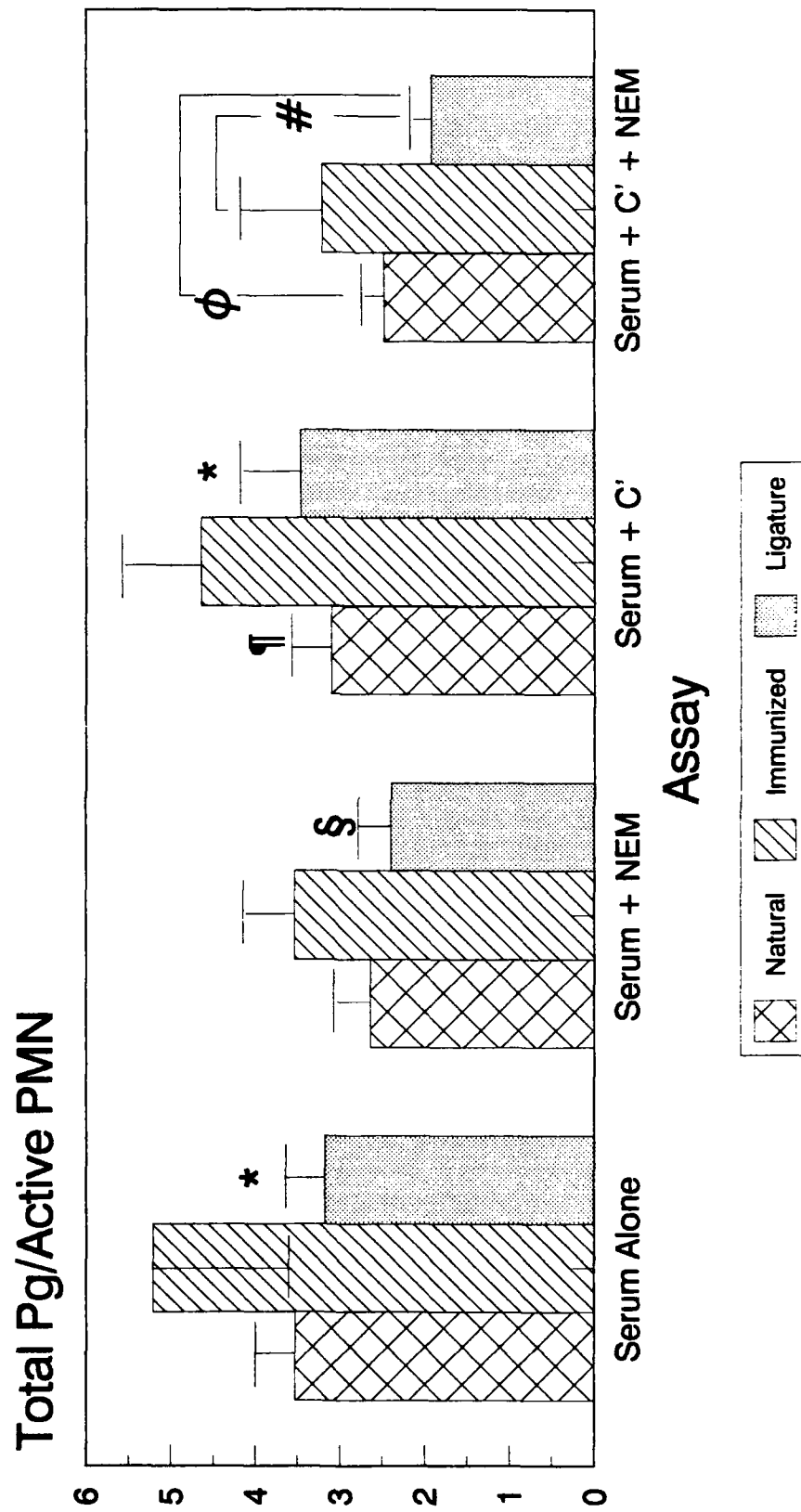
within the periphery of the active PMNs in the NEM trials were uniformly small (Figures 31, 32).

Experiments were also conducted to determine the effect of incubation time on the ingestion/attachment and killing interactions between *P. gingivalis* and PMNs in the presence of serum plus complement. The same variables as discussed above were collected, noting not only vital but non-vital cells at time 0, 15, 30, and 60 minutes. This study was conducted using one serum of each type, with one set of normal PMNs. While sufficient data were not collected for statistical analysis, observational critiques of the outcomes can be discussed.

The results showed that (Figure 33) with increasing incubation time the immunized serum continued to enhance the ingestion of bacteria by active PMNs, whereas the control and natural serum appeared to plateau after 15 minutes. The ligature serum had the least activity of any of the conditions. All experimental conditions revealed little to no killing activity for *P. gingivalis* after ingestion by PMNs through the 30 minute time period. The increased killing detected for the control and ligature serum at 60 minutes may represent artifactual data resulting from the nonspecific staining of nuclear material from lysed PMNs rather than non-vital bacteria. The percentage of active PMNs (with *P. gingivalis* attached or ingested) over time (Figure 34) showed a similar response curve for all sera, except for the control group assays, which peaked earlier. Again, the ligature serum showed the least activity in this assay. In general, the peak level of PMN activity occurred by 30 miuntes except for the immunized serum which revealed increasing activity through 60 minutes.

FIGURE 31 *P. gingivalis* Ingested per Active PMN Actual Ingestion versus NEM Control with Nhp Serum Alone. This graph, on the y-axis, presents the total number of *P. gingivalis* inside the periphery of the PMN as observed under fluorescent microscopy with serum alone. The method of antibody induction is presented on the x-axis. Each bar is stacked with the number of *P. gingivalis* that were found within the periphery in the NEM controls on the bottom and the number of *P. gingivalis* actually ingested on the top part of the bar. T represents the standard deviation for each group.

P. gingivalis Ingested per Active PMN
Actual Ingestion versus NEM Control
with Nhp Serum Alone

Total Pg Inside/Active PMN

Method of Antibody Induction

Legend: ■ NEM Control ⊠ Actual Ingestion

Categories: Natural, Immunized, Ligature, Control
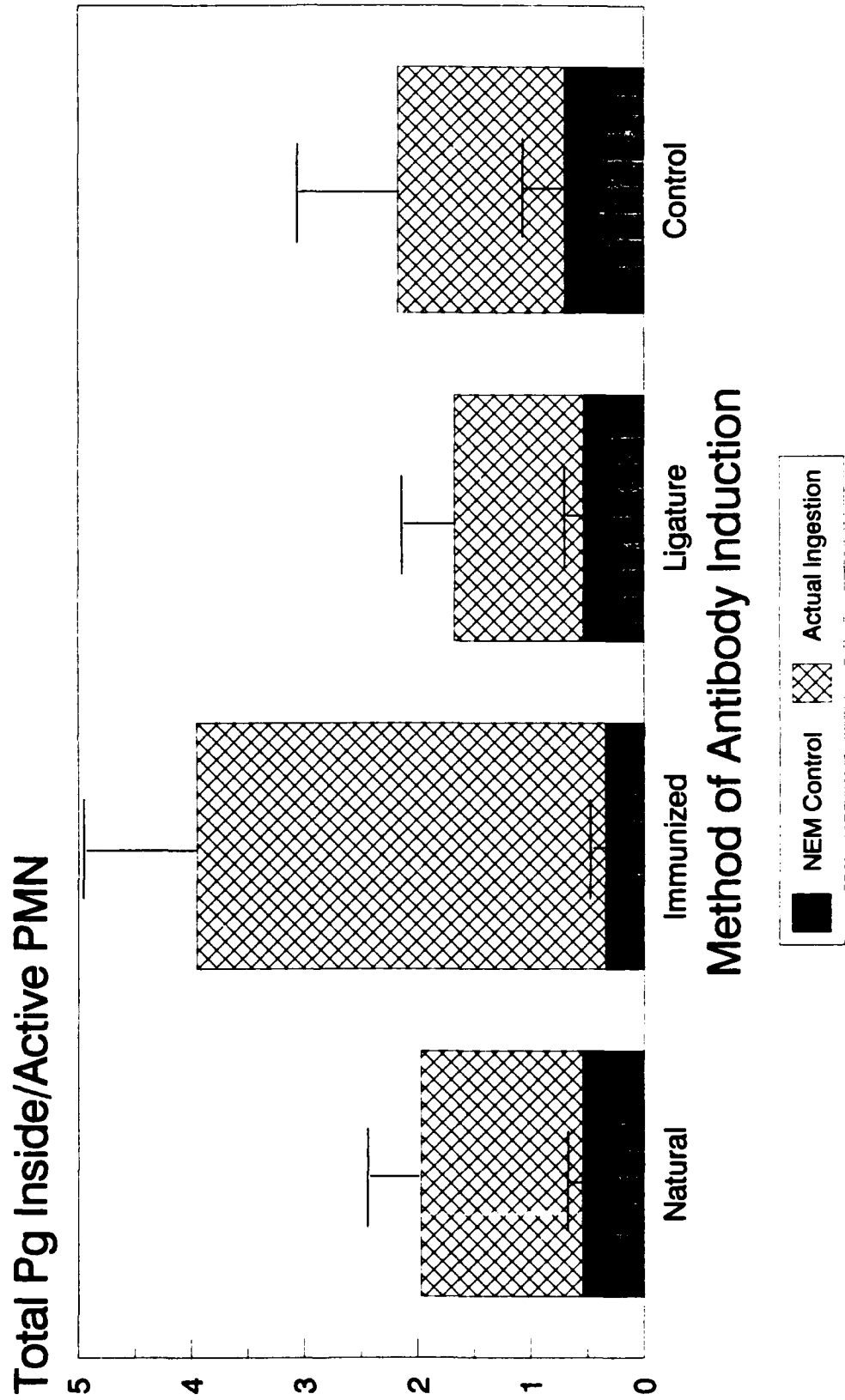
FIGURE 32   *P. gingivalis* Ingested per Active PMN Actual Ingestion versus NEM Control with Nhp Serum plus Complement. This graph, on the y-axis, presents the total number of *P. gingivalis* inside the periphery of the PMN as observed under fluorescent microscopy with serum plus complement.   The method of antibody induction is presented on the x-axis. Each bar is stacked with the number of *P. gingivalis* that were found within the periphery in the NEM controls on the bottom and the number of *P. gingivalis* actually ingested on the top part of the bar.   T represents the standard deviation for each group.

P. gingivalis Ingested per Active PMN
Actual Ingestion versus NEM Control
with Nhp Serum plus Complement
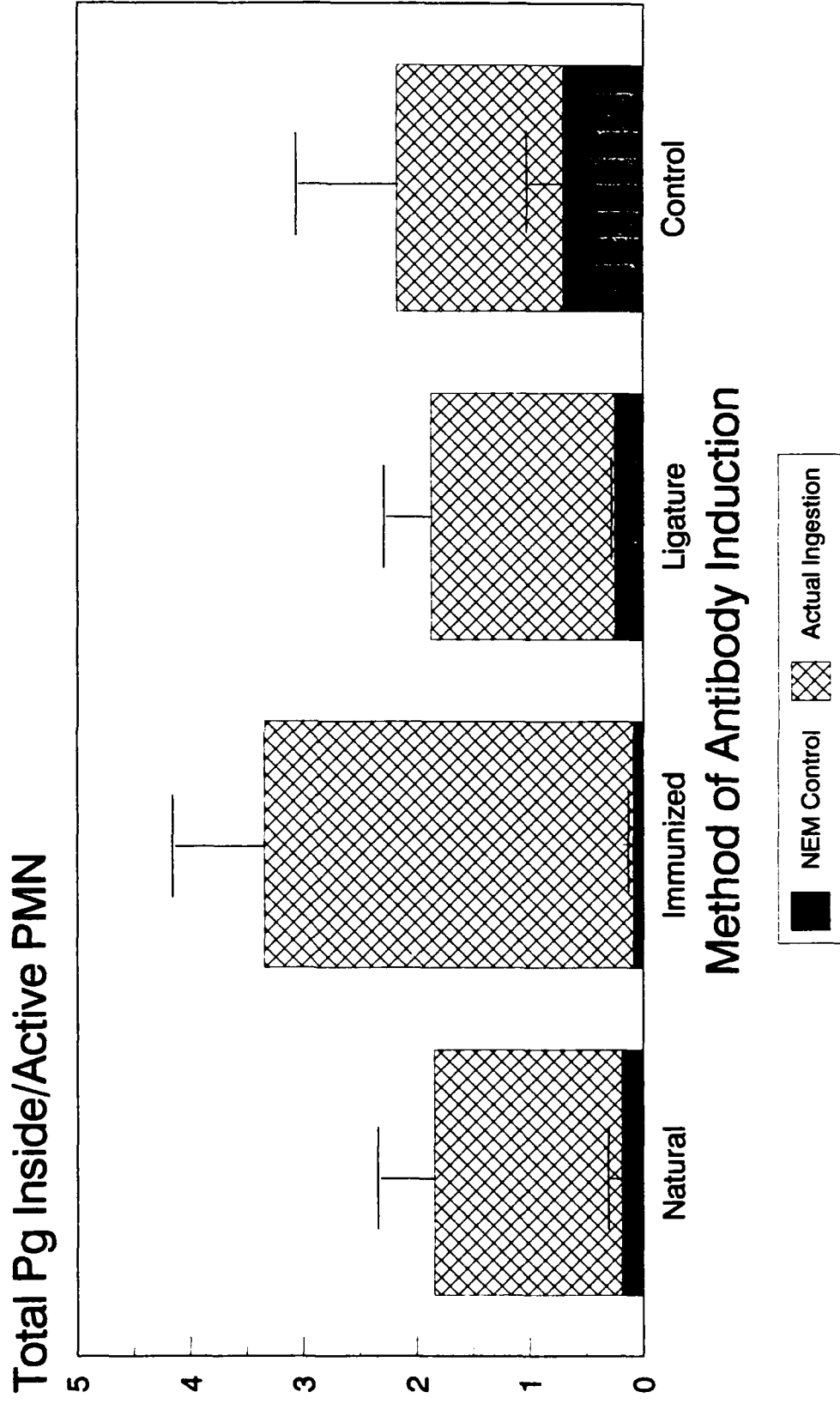
FIGURE 33 Kinetics Assay. Total Vital *P. gingivalis* (attached or ingested) per Active PMN. The top graph presents, on the y-axis, the total number of vital *P. gingivalis* either attached or ingested per *P. gingivalis* positive PMN (Active) over an incubation period of 60 minutes, with readings at time 0, 15, 30 and 60 minutes. All of the assays were performed with complement. The control used PMNs and *P. gingivalis* only, the natural group was represented by serum #11, the immunized group by serum #5, and the ligature group by serum #15.

Kinetics Assay. Total Non-Vital *P. gingivalis* (attached or ingested) per Active PMN. The bottom graph presents, on the y-axis, the total number of non-vital *P. gingivalis* either attached or ingested per *P. gingivalis* positive PMN (Active) over an incubation period of 60 minutes, with readings at time 0, 15, 30 and 60 minutes. All of the assays were performed with complement. The control used PMNs and *P. gingivalis* only, the natural group was represented by serum #11, the immunized group by serum #5, and the ligature group by serum #15.

Kinetics Assay

Total Vital P. gingivalis (attached or ingested)
per Active PMN

P. gingivalis per Active PMN

Time in Minutes

Control	Natural	Immunized	Ligature
PMN + Pg	Serum #11	Serum #5	Serum #15
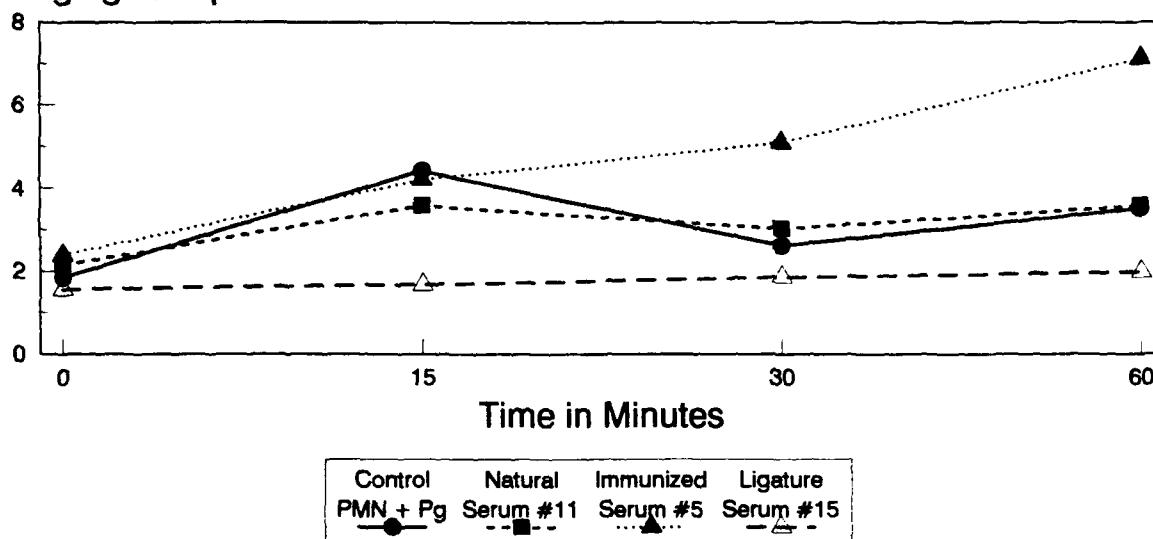
Assay performed with Complement

Kinetics Assay

Total Non-Vital P. gingivalis
(attached or ingested) per Active PMN

P. gingivalis per Active PMN

Time in Minutes

Control	Natural	Immunized	Ligature
PMN + Pg	Serum #11	Serum #5	Serum #15
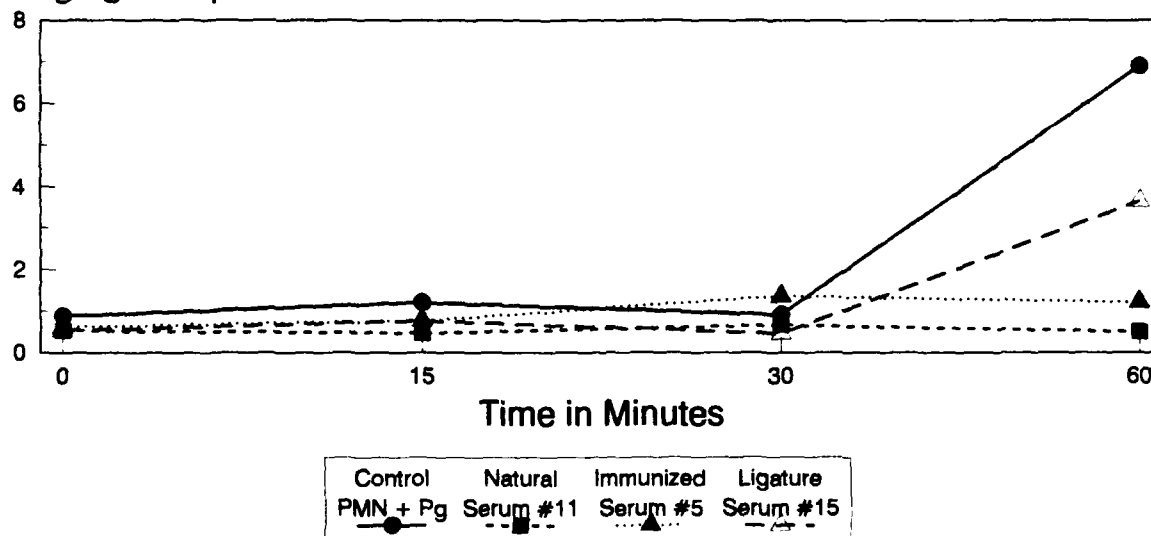
Assay performed with Complement

FIGURE 34   Kinetics Assay Percentage of PMNs Active Against *P. gingivalis* (Pg Attached or Ingested).   This graph, on the y-axis, presents the percentage of *P. gingivalis* positive PMNs (Active i.e. had *P. gingivalis* either attached or ingested) over an incubation period of 60 minutes with readings at time 0, 15, 30 and 60 minutes. All of the assays were performed with complement.   The control used PMNs and *P. gingivalis* only, the natural group was represented by serum #11, the immunized group by serum #5, and the ligature group by serum #15.

Kinetics Assay

Percentage of PMNs Active Against P. gingivalis

(Pg Attached or Ingested)

Percentage of Active PMNs

Time in Minutes

| Control | Natural | Immunized | Ligature |
| PMN + Pg | Serum #11 | Serum #5 | Serum #15 |
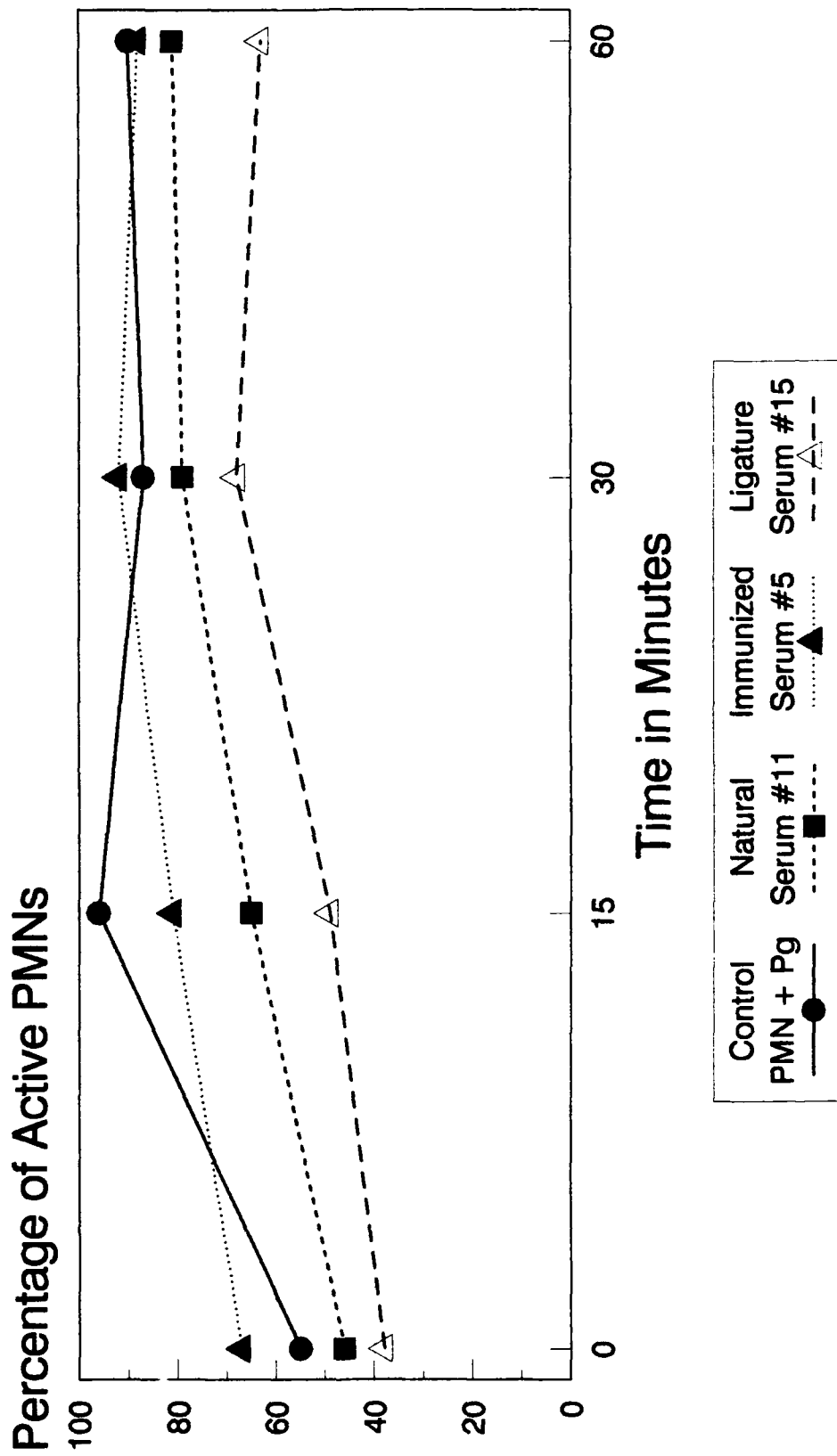
Assay performed with Complement

E.   Functional Tests; Correlations

The results of three tests of functionality have been presented as meaned data for sera derived from nonhuman primates under different experimental conditions. The next set of analyses was to compare these tests among individual sera to assess correlations or associations of functional capacities. Figure 35 presents the percentage of direct killing of *P. gingivalis* and the nanomoles of superoxide released by PMNs as related to the titer of IgG specific for *P. gingivalis*. The immunized sera produced a higher level of both functional activities; however, these two tests were not parallel in their level of activity across the full spectrum of IgG titers. In particular there was a disparity for the three sera at N8.05, N11.49 and N18.13 where the level of direct killing activity was quite high, but the production of superoxide anion was low. Figure 36 compares the percentage of direct killing of *P. gingivalis* with the total number of *P. gingivalis* attached plus ingested per active PMN. A trend reflecting the mean data was seen, in that the immunized sera had a higher level of both activities. However, when comparing the results of the nonimmunized IgG antibody the observation was made that while certain sera acted effectively in direct killing (i.e. N8.05 and N11.49) they did not enhance the attachment/ingestion of *P. gingivalis*. Finally, figure 37 compares the nanomoles of superoxide produced and the number of *P. gingivalis* attached/ingested per active PMN. In this comparison a near parallel response curve is seen between these two tests. This suggests that there may be some relationship between the type of antibody that affects the amount of superoxide anion produced and the number of *P. gingivalis* attached/ingested per active PMN.

All three tests were then normalized to represent one third of the total test activity determined from these assays (Figure 38) and expressed relative to individual

FIGURE 35 Correlation of Superoxide Production and Direct Killing of *P. gingivalis* as Represented by Individual Serum Responses. This graph shows the results of two functional tests, the percentage of direct killing with serum plus complement on the left y-axis and the nMoles of superoxide released in the presence of serum plus complement on the right y-axis. The 18 individual sera are represented on the x-axis by the amount of IgG antibody specific for *P. gingivalis* in ELISA units. The individual sera are marked to identify the subset of sera to which they belong: N=natural, L=ligature, i=immunized.

Correlation of Superoxide Production and Direct Killing of P. gingivalis as Represented by Individual Serum Responses

Percentage of P gingivalis killed

nMoles of Superoxide

ELISA UNITS OF IgG
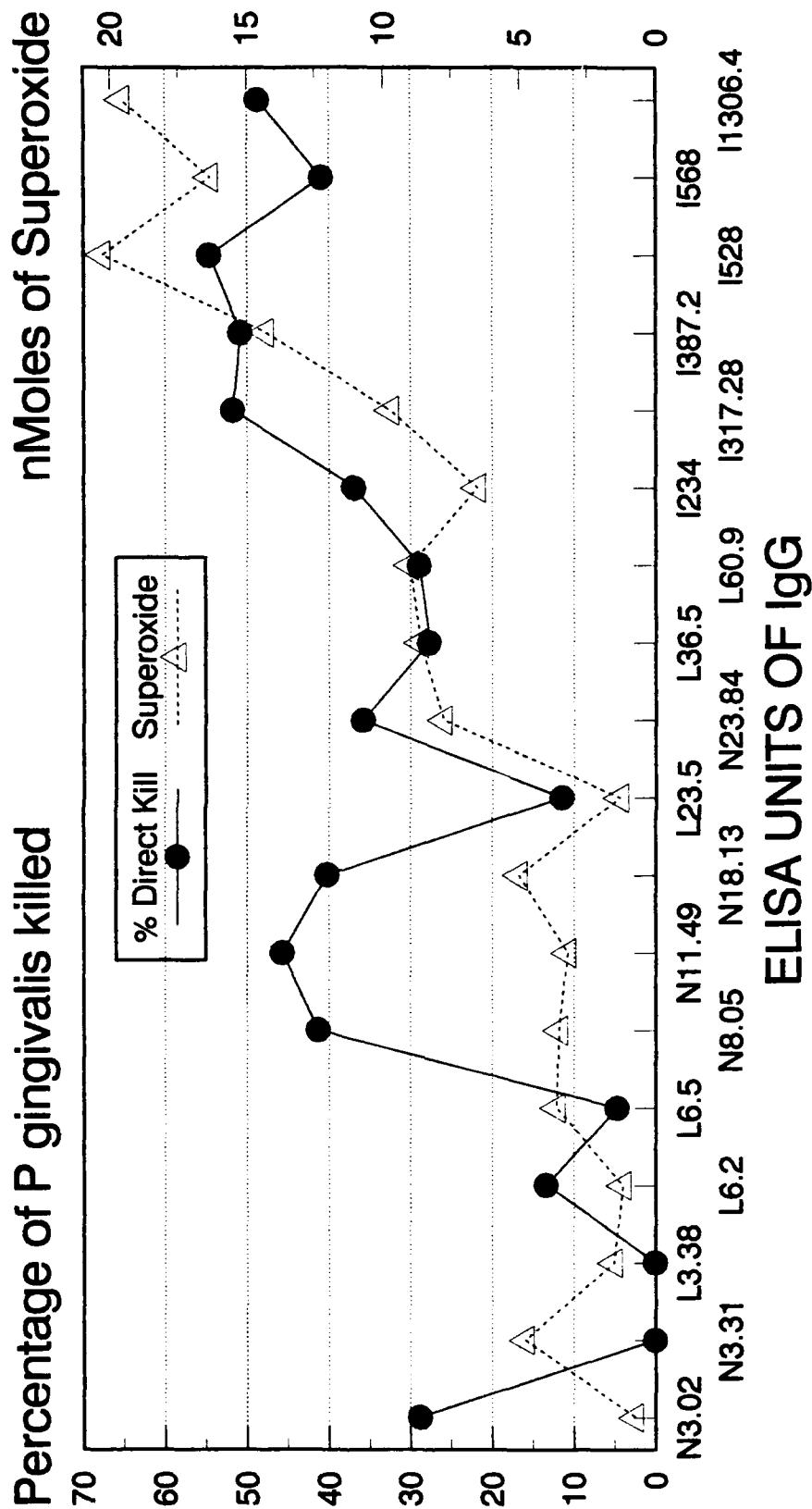
Percentage of Pg killed in assay with human C'.
nMoles of Superoxide Produced with human C'.
N=Natural, L=Ligature, i=Immunized

FIGURE 36 Correlation of Pg Attached + Ingested/Active PMN and Direct Killing of *P. gingivalis* as Represented by Individual Serum Responses. This graph shows the results of two functional tests, the percentage of direct killing with serum plus complement on the left y-axis, and the number of *P. gingivalis* attached plus ingested per active PMN with serum plus complement on the right y-axis. The 18 individual sera are represented on the x-axis by the amount of IgG antibody specific for *P. gingivalis* in ELISA units. The individual sera are marked to identify the subset of sera to which they belong: N=natural, L=ligature, i=immunized.

Correlation of Pg Attached+Ingested/Active PMN
and Direct Killing of P. gingivalis as
Represented by Individual Serum Responses

FIGURE 37 Correlation of Pg Attached + Ingested/Active PMN and Superoxide Production as Represented by Individual Serum Responses. This graph shows the results of two functional tests, the nMoles of superoxide released in the presence of serum plus complement on the left y-axis and the number of *P. gingivalis* attached plus ingested per active PMN with serum plus complement on the right y-axis. The 18 individual sera are represented on the x-axis by the amount of IgG antibody specific for *P. gingivalis* in ELISA units. The individual sera are marked to identify the subset of sera to which they belong: N=natural, L=ligature, i=immunized.

Correlation of Pg Attached+Ingested/Active PMN and Superoxide Production as Represented by Individual Serum Responses
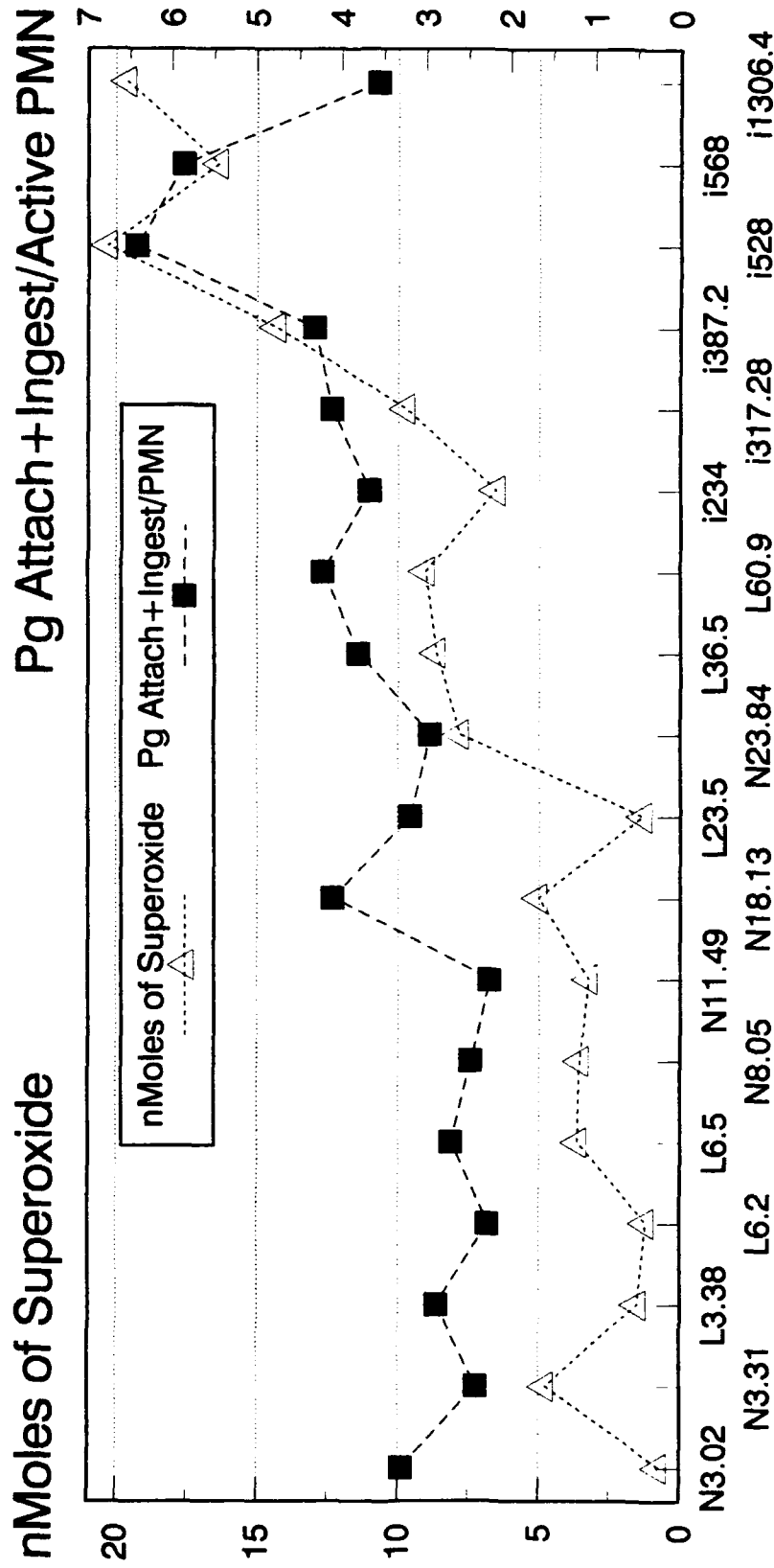
nMoles of Superoxide Produced with human C'
Pg Attached+Ingested/Active PMN with human C'
N=Natural, L=Ligature, i=Immunized

FIGURE 38 Antibody Functional Tests Evaluated for 18 Individual Nhp Sera. This graph shows all three functional tests at one time. The percentage of total functionality is shown on the y-axis after all three tests were normalized to represent one third of the total activity. The red bar represents the percentage of direct killing. The blue bar represents the nMoles of superoxide released from neutrophils in the presence of *P. gingivalis*. The yellow bar represents the number of *P. gingivalis* attached plus ingested per active PMN and is one third of the total functionality. The yellow bar has been further subdivided into the pure yellow area as the number of *P. gingivalis* attached per active PMN and the crosshatched area as the number of *P. gingivalis* ingested per active PMN. All assays were performed with nonhuman primate sera and complement. The 18 individual sera are represented on the x-axis by the amount of IgG antibody specific for *P. gingivalis* in ELISA units. The individual sera are marked to identify the subset of sera to which they belong: N=natural, L=ligature, I=immunized.

Antibody Functional Tests
Evaluated for 18 Individual Nhp Sera
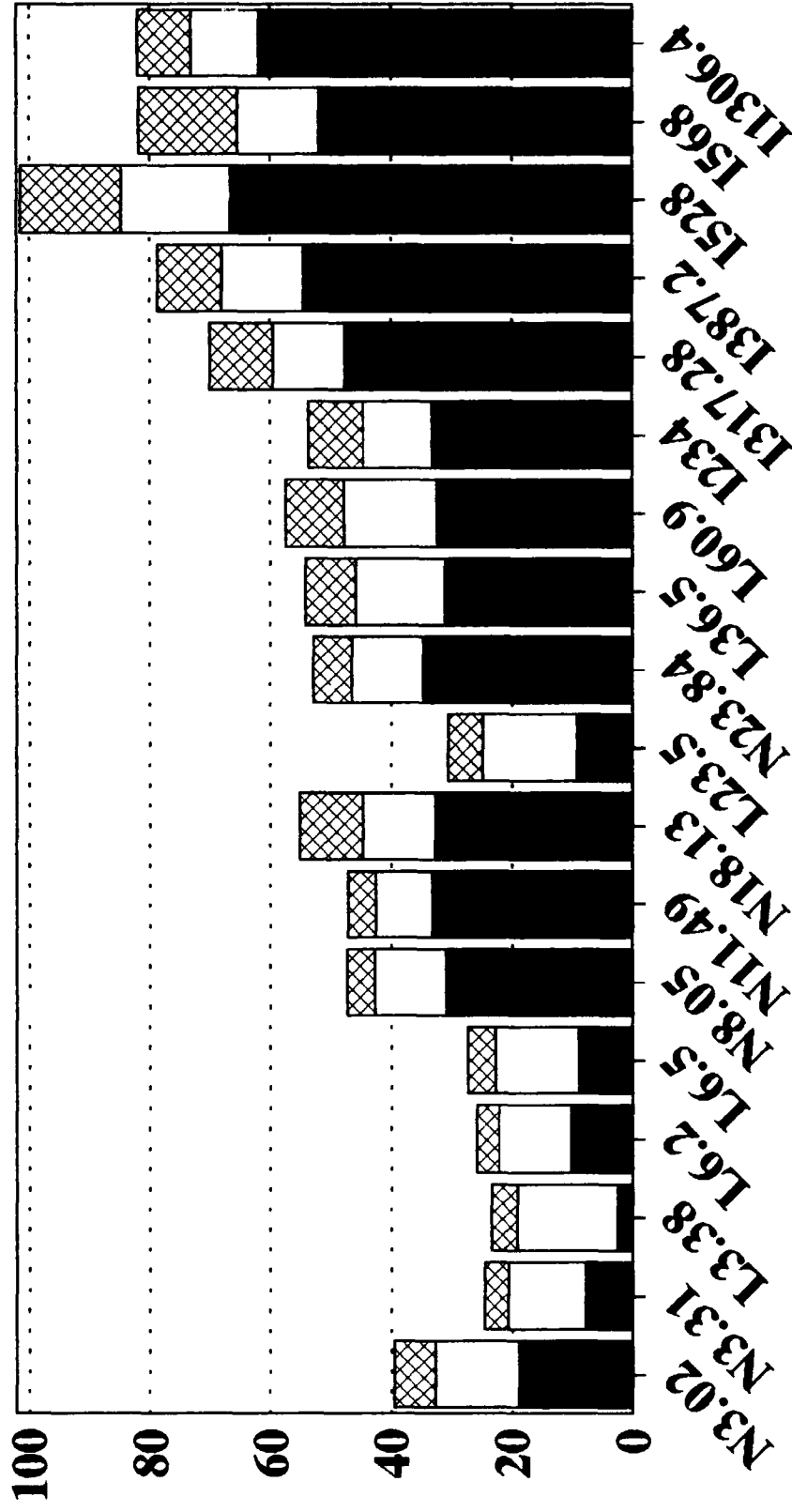
Titer of IgG Antibody to P. gingivalis

Legend: ■ Direct Kill ■ Superoxide □ Pg Attached/PMN ⊠ Pg Ingested/PMN

Assay. with Nonhuman Primate Serum plus Complement

titers of IgG antibody to *P. gingivalis.* It was observed that while the immunized sera had a higher level of overall activity, these functions were not linearly related to IgG antibody levels. Furthermore, a wide variation in the expression of these functions were noted among the sera. This data presentation also reinforce that the number of *P. gingivalis* attached remained fairly constant, but that the number of *P. gingivalis* ingested by the PMNs was related to antibody functional activity in this test.

IV. DISCUSSION

The interactions between the host and pathogens is complex in periodontal disease, involving a great number of variables for disease initiation, progression and maintenance. The balance and absolute number of bacterial species vary from host to host as does the pathogenicity of the microorganisms. Likewise, resistance to disease varies between patients and may depend upon a number of immunological factors. Deciphering the interaction between host and pathogen requires a controlled environment and the ability to manipulate clinical variables. The nonhuman primate appears to provide a good model for studying periodontal disease, especially as it relates to the pathogen, P. gingivalis. Exploring the functional capabilities of the immune system, specifically the immunoglobulins, present in whole serum, should better define the role of the immune system in host defense against this disease.

A. Direct Killing Assay

Bacterial resistance to the cidal activity of serum is believed to be associated with the virulence of the bacteria, since some of the more virulent species appear to resist serum killing better than nonvirulent ones. A functional test assessing the ability of serum to directly kill bacteria will evaluate two parameters, the level of host defense and the resistance of the bacteria.

There does not seem to be a consensus as to which set of conditions best measure the *in vitro* bactericidal activity of serum. There are number of factors to consider in designing this type of assay. The growth phase of the bacteria appears to be important, since it has been observed that *E. coli* and other gram negative bacteria are more readily susceptible to serum killing when harvested in the early logarithmic phase as compared to either the lag or stationary phases (Davis and Wedgwood, 1965). This study used late log phase of growth for all of the assays, which appeared to be optimal for testing the serum bactericidal activity against *P. gingivalis.*

Another area of concern is the pH and ionic balance of the solution used for bactericidal evaluation. The use of solutions containing phosphates such as phosphate buffered saline, can cause the bacteria to clump, making accurate enumeration difficult, both in plating and in microscopic observation of the bacteria. For optimal activation of complement, certain buffering and ionic concentrations are required. The gelatin-veronal-buffered saline solution plus Mg^{2+} and Ca^{2+} ions, used in this study was the same solution used for complement fixation of sheep red blood cells, and helped to optimize the complement activity without adversly affecting the vitality of the gram negative organisms (Taylor, 1983).

Acquiring the proper bacterium to serum ratio was another critical area for analyzing the direct killing properties of the serum, since an excess of immunoglobulin can have an inhibitory effect upon bacteriolysis (Taylor, 1983) and an abundance of bacteria can result in no killing activity (Okuda, et al., 1986). The results from this study revealed a plateau of around 50% killing for some individual sera in both the high and low titer IgG groups.

Five major possiblities may explain why those sera achieved no higher than 50% killing in the presence of complement. The first was that the sera with low IgG titers may have been performing at their maximum level in the direct killing of bacteria and would have achieved no greater killing under altered conditions. Another possibility, was that an inadequate titer of complement was used, restricting the level of killing due to insufficient complement proteins. A third possibility for restricted killing in the high titer serum was an inhibition of killing due to an excess of IgG immunoglobulin (Taylor, 1983). A fourth reason may may have been an inadequate amount of time allowed for the reaction to occur, since other studies testing bactericidal activities of serum allowed 60 minutes for their first evaluation. The last reason may have been related to the ratio of IgG subclasses present, since IgG1, and IgG3 are well known to have some complement activity; however, IgG2 and IgG4 generally lack this property, but would be included in the titer of IgG present.

One study (Van Der Zee, et al., 1986) suggested that the presence of IgG4 acts to protect bacteria from the biological effects of the IgG subclasses which fix complement; therefore, the ratio of IgG subclasses may be important in defining direct killing activity. Wilton et al. (1992) in a study looking at IgG subclasses in adult periodontitis found a correlation between disease activity and high levels of IgG4 antibodies, suggesting that this might be a marker for patients with active disease. Giardino (1991) in examination of nonhuman primate serum response to *P. gingivalis* found an increase in the level of IgG4 post immunization, and a drop in IgG4 from baseline in a group of periodontally ligated animals. These studies suggest that there is no clear pattern of IgG subclass responses, reinforcing the concept that there may be diverse host responses to the clinical disease known as periodontitis.

Some other explanations for the plateau of direct killing include the presence of IgA immunoglobulin in the immunized group sera since Giardino (1991) found a rise in IgA post immunization. The level of IgA becomes important because *P. gingivalis* has been shown to possess IgA proteases capable of cleaving IgA (Grenier, 1992). This cleavage not only results in the inactivation of IgA, but the presence of an IgA Fab-alpha component which has been shown to have a protective function by coating the bacteria. This fragment appears to compete for the same antigen site used by the IgG molecule, but without any biologic function of its own. This effectively blocks other immune effector systems such as complement activation (Russell, et al., 1989).

The role of IgM in the bactericidal activity cannot be ruled out, since numerous studies have noted the ability of IgM to function with 70 to 560 fold greater activity toward killing bacteria than IgG, on a per weight basis and only one IgM molecule is needed to activate complement instead of the two adjacent molecules required by IgG. The level of serum IgM present in this group of monkeys post ligation was very low, and probably played only a limited role in complement activation in this assay. The determination of which elements are most responsible for direct killing needs to be as ascertained as well as determining the overall functional activity of the serum.

Overall, the sera containing higher levels of IgG outperformed the lower titer sera; however, the association was not linear, perhaps for some of the reasons listed above. Within the natural and ligature sera some samples performed nearly as well as the immunized group for direct killing, suggesting a heterogeneity of function despite similar IgG titers. While it may be interesting to design experiments to answer which parameters restricted the direct killing, and which factors were most responsible for direct killing within each serum, the use

of whole serum in this assay, more closely approximates the *in vivo* serum conditions present in the host and serves as a valid test for comparing these immune responses.

Another area of controversy when performing a bactericidal assay is the means of assessing bacterial viability after exposure to potentially lethal conditions. One common method of determining viability is to plate the bacteria with serial dilutions and count the resultant growth of colonies. The problems associated with this method are the plating efficiency of the bacteria and the agglutination of bacteria during the bactericidal reaction leading to an underestimation of the survival rates of the bacteria. Another assumption of the plating method, is that the rate of bacterial replication does not vary after exposure to potentially damaging solutions of serum and complement. It may be possible that cells damaged, but not killed by the serum--complement exposure, would concentrate for some time on repair of the cell before resuming replication.

The above reasons add strength to the methodology of using dyes to assess viability ratios during a bactericidal assay. DAPI has the ability to complex with the AT, AU or IC clusters of double-stranded deoxynucleic acids (Manzini, et al., 1983) allowing clear visualization of the bacteria with fluorescent microscopy. Moreover, propidium iodide, which penetrates into the nonvital cells, provides a clear distinction between cells with intact cell membranes and those with terminal damage.

A possible conclusion from these results was that increased levels of serum IgG were more effective in direct killing and thus may be more protective by direct killing. Other studies have also shown an association between

increased levels of IgG and increased protection. In the murine model, increased levels of IgG1, IgG2b, and IgG3 antibodies specific for pneumococcal capsular polysaccharides have proven to be highly protective (Briles, et al., 1989) against pneumonia. In another study, human serum with a high titer of IgG specific for LPS, was injected into the murine model. The serum provided protection to the host against an otherwise lethal injection of *Salmonella typhimurium* (Fomsgaard and Galanos, 1989). Dunn et al. (1984) immunized a horse with a bacterial outer membrane lipopolysaccharide (LPS) common to many gram negative bacteria with a resultant elevation in IgG titer that proved to be protective against lethal doses of gram negative bacteria in the murine model. In that study the anti-outer membrane LPS IgG Fab fragments were tested alone and were found to be just as protective as the whole IgG molecule, suggesting it protected the host by ar titoxin mechanism. Another study (Okuda and Kato, 1991) immunized mice with *P. gingivalis* 16-1. These authors found that the immunized mice had a significant increase in the level of IgG specific to *P. gingivalis* and had a reduction in spreading abscess formation with a reduction in lethality. Abscess formation still occurred at 4 to 10 weeks in the immunized mice, resulting in a localization but not elimination of the infection.

If the production of greater amounts of IgG are protective then the absence of this immune component should increase the chances of disease progression. Beard, et al. (1986) examined a group of children with recurrent or severe respiratory infections looking at neutrophil chemotaxis, bactericidal, fungicidal, and quantitative iodination activity as well as complement function. He found that 9 of 22 children had at least one IgG subclass level two standard deviations below normal and two children presented with a deficiency of more than one IgG subclass. They found the IgG1 subclass was predominantly lower

than the other subclasses of IgG. The authors' concluded that a deficiency of IgG was increasing the susceptibility of the host and that increasing the titer of IgG in these patients might reduce the infection rate and severity of disease.

The presence of immunoglobulin as well as an adequate level of complement, has been shown to be important in a direct killing assay. Numerous studies (Schenkein, 1987, 1988a) have shown that certain strains of P. gingivalis have the ability to degrade complement proteins, disrupting the mechanism of complement activation. It has also been shown that P. gingivalis has the ability to deplete complement from its cell surface by activating C3 in the environment through the expression of a complement factor D-like activity which in turn provides protection to the cell (Schenkein, 1991). This appears to be an important virulence factor associated with this bacteria, and may be important in avoiding host immune defenses.

Numerous studies (Gregory, et al., 1992) have now shown that the trypsin-like protease produced by P. gingivalis, is active against all 4 subclasses of IgG, IgA1, IgA2, IgM, IgD and IgE, and it appears to be a molecule with an approximately 80 kDa molecular mass (Grenier, 1992). Grenier and Belanger (1991) examined the proteolytic properties of outer membrane vesicles from P. gingivalis and found that these vesicles contained proteases which not only inactivated serum components against P. gingivalis, but also reduced serum complement factors helping to protect other bacterial species. After the discovery of these potent proteases Harper and Curtis (1990) examined P. gingivalis to elucidate methods by which this bacteria did not autodegradate in the presence of such high levels of protease. They found that P. gingivalis had the ability to concentrate high levels of hemin, and proposed that this served as a protective mechanism, preventing autodegradation of the bacterial cell wall.

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The results of the present study reinforces the conclusions of the studies noted above, since opsonization with serum containing immunoglobulin for thirty minutes prior to the addition of complement resulted in no increase in direct killing activity over serum alone. However, when both serum and complement were added simultaneously a significantly greater amount of direct killing occurred, suggesting that the classical complement cascade was initiated before the immunoglobulins were neutralized *P. gingivalis* proteases. Therefore, one could recommend that in an assay testing the direct killing ability of serum and complement in the presence of *P. gingivalis*, both reagents should be added simultaneously to give optimal results.

One early study tested (Sundqvist and Johansson, 1982) pooled human serum against *Bacteroides melaninogenicus* subsp. *melaninogenicus* strains ATCC 15930 and ATCC 25845. They incubated the bacteria for 60 minutes with whole serum and found as little as 2% viability of the bacteria via a plating method. A substantial amount of variability existed between the two strains when absorbed serum, and serum lacking classical complement components, were tested. Serum heated to 56°C for 30 minutes was also tested under the same conditions and resulted in no direct killing. The authors were not able to show any correlation between IgG titer and the bactericidal effectiveness of the serum. They speculated on the differences in the resistance to killing between strains of bacteria and concluded it could be due to differences in the bacterial cell wall.

Okuda, et al., (1986) compared serum from healthy adults, adult periodontitis patients and immunized rabbits in a bactericidal assay against *B. gingivalis* (*P. gingivalis*) 381. They also used a plating method for testing viability of the bacteria. The incubation times of 60 to 180 minutes showed that

most killing took place in the first 60 minutes with little significant killing occuring after that time period. The healthy adult serum showed little killing activity, the periodontitis serum reduced the bacterial counts from 10^7 to 10^5, and the hyperimmunized rabbit serum essentially killed all bacteria in 60 minutes. No correlation was found between the titer of immunoglobulin and the activity of the serum against the bacteria.

The data from this study agrees with earlier studies in that no linear correlation existed between IgG antibody titer and direct killing; however, the immunized sera, while containing a variable amount of IgG, did consistently out perform the natural and ligature sera. Another important result of this study is that the meaned results of the ligature group showed less direct killing activity than the natural group. While it is tempting to attribute a loss of functional ability to the induction of periodontitis, the sera were not selected longitudinally from the same animals; therefore, it is not known if the ligature sera originally had a higher level of activity prior to disease progression.

When the results of direct killing in the absence of complement were examined, a relatively high percentage of bacteria became non-vital. This was a major difference between this study and previous studies. Sundqvist and Johansson, (1982) and Okuda, et al., (1986) found no direct killing in human serum after heating to inactivate complement. An explanation for this apparent difference could be the presence of nonspecific serum proteins in nonhuman primates (i.e. defensins and lysozymes), since immunglobulins alone have not been shown to have any direct bactericidal activity in the absence of complement. It appears that these proteins have the ability to kill bacteria in the absence of complement. This has one potential benefit, in that the number of bacteria could be decreased without increasing the release of chemotactic

factors, which occur during complement degradation. This proposed chain of events may result in increased host resistance and explain the natural resistance to periodontal disease in this animal model. It was also interesting to note that the ligature group appeared to lack most of this ability compared to the natural and immunized groups. Therefore, the best explanation for this difference is probably specific to this animal model. While the reporting of bactericidal activity without complement appears unique to this study, a more effective killing of bacteria occurred in the presence of complement as would be expected, demonstrating the synergistic activity of immunoglobulin and complement.

B.   Superoxide Production by Neutrophils

The ability of neutrophils to eliminate bacteria can be grouped into two classes based upon the availability of oxygen in the local environment (Mandell, 1974). In the presence of oxygen, neutrophils, monocytes, macrophages and eosinophils (Cohen and Chovaniec, 1978) have the ability to produce superoxide, which at neutral pH, results in the production of H_2O_2. Under normal conditions, the neutrophil encloses the foreign particle within a phagocytic vesicle and a production of metabolites is initiated which are eventually released into the vesicle. A lag time of up to several minutes may occur between the binding of the particle and the release of destructive metabolites such as superoxide.

An efficient method of measuring intracellular superoxide production has not been developed; therefore, a method of getting the neutrophil to produce

superoxide extracellulary was accomplished by exposing the neutrophil to cytochalasin B, a fungal metabolite. This prevented the closure of the phagocytic vesicle by interfereing with the internal cytoskeletal components (Hartwig and Stossel, 1976). By preventing the internalization of bacteria, an extracellular release of superoxide was produced, measured by the change in absorbance of cytochrome c.

A number of studies in the literature have examined the release of superoxide from neutrophils using a variety of experimental conditions. In a majority of these studies a standard stimulus such a N-formylmethionyl-leucyl-phenylalanine (fMLP) was used to evoke a response from the neutrophil. One of two experimental conditions were usually used, either healthy neutrophils were exposed to an experimental reagent and then stimulated, or the neutrophils were harvested from patients believed to have some altered immunologic condition, and assessed for metabolic functions, such as superoxide production. There are a few studies that have used opsonized particles to detect the response of the neutrophil. Ohman, et al. (1992) used opsonized yeast particles to assess the receptor-mediated functions of the neutrophil through chemiluminescence, phagocytosis and degranulation. Another study did not use an opsonin (Mangan, et al., 1989) but did use an oral bacteria, Fusobacterium nucleatum, to test the lectinlike interactions with healthy human neutrophils by measuring the activation of neutrophils using a number of tests including superoxide production.

This study differed from others in that a standard stimulus such as fMLP was not used. Instead, the experimental protocol was designed to test the influence of whole, nonhuman primate serum on the interaction between a specific bacteria, P. gingivalis and human neutrophils. One advantage of this method was that the protective/virulence factors of the bacteria were left intact,

providing a more natural interaction with the neutrophil. One criticism of this method might be the variability induced by the presence of bacteria floating in suspension as the absorbance measurements were recorded; however, the same volume of bacteria was added to both the reference and experimental cuvettes, minimizing this experimental error. Another possible concern for this methodology was the use of neutrophils and complement from a human source with nonhuman primate sera. This protocol can be justified; however, due to the conservation of neutrophil surface receptors across higher species of mammals thus validating the use of human neutrophils in this assay. Another justification for the use of human neutrophils was the convenience of harvesting these cells from donors selected to be healthy and free of immunological deficencies.

In this study the total amount of superoxide produced under each set of conditions was interpreted as an indirect measurement of the interaction between the bacteria with the neutrophil. Complement and antibody were both required for optimal neutrophil activation, with little activity occuring in the absence of complement. The production of superoxide was reported two ways: kinetically over a 30 minute time period; and as the total production of superoxide produced in 30 minutes. According to Newberger et al. (1980) neutrophils undergo a lag time of up to several minutes depending on the experimental conditions. The lag time was not represented graphically most of the time because of the time needed to complete the addition and mixing of the final reagent physically located outside the spectrophotometer.

This experimental protocol appears to be a valid mechanism for assessing the influence of serum on the interaction between bacteria and neutrophils. As was noted in the direct killing assay, a great deal of variability was also found in the individual serum responses for contributing to the

production of superoxide. It is interesting to note that some the nonimmunized animals that responded with a high percentage of direct killing were low responders in superoxide production, and likewise, some of the low responders in direct killing induced high amounts of superoxide. The reason for this phenomenon may be attributed to the presence of different subsets of immunoglobulin or antigen specificities, each with associated functions, excelling in differenct areas of host defense.

C. Ingestion, Phagocytosis and Killing by Human Neutrophils

A number of researchers have tried to evaluate the specific interactions between neutrophils and bacteria by assessing the abilities of the neutrophil to attach to, phagocytose and kill the target organism. The ability to attach to the target cell is believed to be the first step in the defensive mechanisms of the neutrophil, followed by ingestion, and finally killing of the target organism within the phagolysosome. Evaluation of these three steps in the clearance of bacteria are important for further elucidation of host immune interactions.

Attachment of bacteria to the surface of the neutrophil can occur under two conditions, with or without the participation of an opsonin. There are two main types of opsonins which can participate in this reaction: antimicrobial antibodies, usually IgG, which bind to the target cell and allow binding of the Fc portion of the antibody molecule to the Fc surface receptor on the PMN; and complement fragments such as C3b and C3bi which bind to the target bacteria and form a complex with the CR1 or CR3 receptors on the PMN. The third level

of binding in the presence of opsonins, occurs where both antibody, and complement fragments combine on the surface of the target cell with a subsequent interaction with both Fc and CR receptors on the neutrophil. This binding is much stronger than either the antibody or complement fragments alone.

A second type of interaction between neutrophils and bacteria occurs in the absence of opsonins and may be through a mechanism involving carbohydrate-binding proteins, collectively known as lectins. Lectins may be present on the surface of either the neutrophil or the target cell. They bind with a complementary sugar on the opposite cell surface in a lock-and-key manner (Ofek and Sharon, 1988). This type of recognition has also led to phagocytosis in certain cases, which has been termed lectinophagocytosis. Mangan et al. (1989) has shown that lectinlike interactions can be strong enough to produce phagocytosis and the release of superoxide from human neutrophils in the presence of *F. nucleatum*.

In this study the number of *P. gingivalis* attached to the surface of the neutrophil remained fairly constant thoughout all experimental conditions (no serum, serum alone, and serum plus complement) with no significant differences noted. The number of neutrophils participating, also remained fairly constant throughout the same set of conditions with approximately 65% having some interaction with *P. gingivalis*. These results suggest that there may be some opsonin independent mechanisms which allow the binding of *P. gingivalis* to the neutrophil surface.

Katancik et al. (1993) evaluated the interaction of *P. gingivalis* with neutrophils with and without anti-*P. gingivalis* IgG positive human serum. Their

results were similar to this study in that the presence of the antibody positive serum did not alter the number of bacteria attached to the neutrophils, as measured by ELISA. Cutler et al. (1991) performed a series of experiments using a similar technique of fluorescent microscopy to enumerate the attached, ingested and killed *P. gingivalis* per PMN. They got up to 100 percent participation of the neutrophils in the presence of serum and complement depending on the strain of *P. gingivalis*. There are two possible reasons they achieved different results. It could have been their methodology, which included a 15 minute incubation period with the opsonins prior to reacting the bacteria with neutrophils, which may have allowed an increased opsonization of the bacteria resulting in a reduction in some of the defensive mechanisms of the bacteria prior to neutrophil exposure. The second reason may have been the particular strain of *P. gingivalis* used in this study since even in Cutler's (1991) study there was significant difference in PMN activity between different strains.

The next area of analysis was the number of *P. gingivalis* ingested per PMN. In this study a significantly greater number of bacteria were ingested in the presence of the immunized sera compared to the other two groups. The results of the kinetics assay showed that one immunized sera continued to cause an increase in the number of *P. gingivalis* phagocytosed up to 60 minutes compared to the natural and the ligature sera. The presence of complement did not seem to have any influence on the number of bacteria phagocytosed.

Nisengard, et al. (1993) found the same trend in testing the opsonic activity of rabbit anti-*P. gingivalis* serum (whole cell or cell fractions). They found that the antisera to the cell fractions had opsonic properties for neutrophil phagocytosis, and that complement did not increase the number of bacteria ingested. Van Dyke et al. (1988) looked at the number of *B. gingivalis* (*P.*

gingivlis) ingested by neutrophils with the addition of human serum, from both healthy and adult periodontitis patients. They found only 7 percent of the neutrophils partcipating under the influence of the adult periodontitis serum, and around 1 percent with the healthy serum. The bacteria to neutrophil ratio in the adult periodontitis serum was only 0.074. In the study by Cutlter et al. (1991) the number of *P. gingivalis* ingested per neutrophil ranged from 2-8 depending on experimental conditions. This range is very close to the results achieved in this study. However, due to the methodology for enumerating bacteria in this study, the ratio may actually be higher than reported. When data collection was performed, the number of PMNs active were counted, but the distinction was not made as to which ones had only *P. gingivalis* ingested, only attached or both; therefore, the number of bacteria per PMN may be actually larger than reported. It appears that IgG specific antibody plays a role in host defense by increasing the interaction between neutrophils and pathogenic bacteria through a mechanism specifically involving the Fc receptor.

The last area is the number of cells that were rendered non-vital once ingested by the PMN. Okuda and Kato (1991) using the murine abscess model found that while neutrophils had ingested large numbers of *P. gingivalis* none of the bacteria were killed. Cutler et al. (1991) found that the number of nonvital bacteria phagocytosed ranged from 20-50 percent depending on experimental conditions. Nisengard et al. (1993) found that in the presence of serum and complement, bacteria viability from neutrophil interaction was reduced 88.5-91.7 percent. The results of the present study are more in agreement with those of Okuda and Kato (1991) in that very few non-vital bacteria were found within the neutrophil. The most likey reason for the differences between these studies may

be due to the use of different strains of *P. gingivalis* and different sources of serum.

The one exception in the present study, to low numbers of ingested bacteria being nonvital, was during the kinectics study where the ligature and control groups showed a large increase in the presence of prodium iodide stained material within the perimeter of the PMN. It appears that this stained material was probably artifactual material, open to interpretation as to its origin. Since only a few trials were run in the kinetics assays, not enough data is available to determine if this was a consistent finding, or to speculate on the specific reasons for this phenomenon.

As with the other functional tests performed in this study there was a great deal of variation between individual sera for ingestion of *P. gingivalis,* but little variability for the number of bacteria attached. It was also interesting to note the graphic representations comparing direct killing and superoxide production appeared unrelated as did the comparison of direct killing and attachment/ingestion. However, when superoxide production and attachment/ingestion assays were compared a loose relationship was revealed with somewhat of a parallel response curve of functionality, suggesting that the subset of antibody responsible for superoxide production, may also be the same subset active in the ingestion of *P. gingivalis.*

D.   Implications Of Antibody In Host Defense

Numerous studies have attempted unsuccessfully to correlate the titer of antibody with either the functional activity of the serum, or the clinical parameters of periodontal disease. The only relationship that consistently appeared was that found by Nakagawa et al. (1990) who showed that a significantly elevated level of anti-*P. gingivalis* IgG antibody in the serum correlated with elevated levels of *P. gingivalis* in the periodontal pocket. This would suggest that the antibody response is not protective in these patients, possibly for a number of reasons, such as inaccessiblity to the infection, lack of antibody specificity, inadequate PMN function or a combination of these (Cutler, et al 1991). It may be that the elevated antibody responses actually provide a protective mechanism to the pathogen, depending on the specific isotype and subclass of antibody being produced by the host. One trend appears throughout all of the research in antibody and functional responses to periodontal disease, and that is the great deal of variabilty between subjects.

The functional tests presented in this study demonstrate that immunization with a formalin killed strain of *P. gingivalis* can produce a serum with increased antibody levels which outperform sera from both natural and ligature induced animals. The variability found in this study may actually prove to be the most important finding, because this may allow an evaluation to be performed which correlates the specific functional test with the clinical response of the animal. For example, it may be that the animals producing sera responsible for higher levels of superoxide where actually more damaging to the tissues of the periodontal pocket resulting in more disease progression.

E.   Future Directions Relating Antibody and Functional Tests

One of the first followup analyses will be to look at the clinical data from each of the nonhuman primates in the original study. Based on their individual disease progression one could attempt to correlate attachment loss with either individual or a combination of functional tests. Another study could be conducted, using the same set of sera from these nonhuman primates, to evaluate the changes in functionality over time, attempting to determine if there was actually a loss of functionality in those animals after induction of periodontal disease. Following this, one could attempt to define a relationship between functional activities and specific epitopes on the bacteria. In other words, was there a common epitope for the antibody performing well in direct killing assays and then was this function important in host defenses against periodontal disease.

From this point a study could be conducted longitudinally on sera from humans with periodontal disease looking at the functional activity of whole serum over time to various putative pathogens. An attempt could then be made to correlate various forms of periodontal disease with patterns of functional deficiency, looking for associations that have not been possible with antibody titers alone. Through the use of functional tests, as described in this study, as well as additional tests, such as assays to determine the role of serum in preventing adherence of bacteria to epithelial cells, one might be able to classify the heterogenous group of patients now diagnosed as having adult periodontitis. This could enable one to predict those patients most at risk for initial disease and those most at risk following periodontal therapy as well as those that might not

respond to standard therapeutic measures. If a correlation among these tests and disease progression was found, it is possible that a predictive diagnostic test for serum could be developed based upon the serums response to specific epitopes. If such a set of epitopes were found, the next logical step would be a modification of the host immune response through vaccination and subsequent clinical follow-up.

V. CONCLUSION

The presence of IgG appears necessary for adequate host defense, and higher titers appear to protect better in most infections, especially when directed against specific bacteria causing the infection. Complement also appears essential in providing optimal host defense against bacterial pathogens. The virulence of *P. gingivalis* appears to be dependent upon a number of factors. The production of proteases directed at immunoglobulin and complement, appear very critical to infection, especially since they can be packaged in outer membrane vesicles. The syngerism that exists between *P. gingivalis* and other bacteria against host defenses, further complicates the investigation of attachment loss in periodontal disease.

In order to clarify the importance of the host immunoglobulin levels, the functional ability of the immunglobulins must be defined. There are a variety of functions proposed for immunoglobulin IgG which may play a role in host defense and/or host tissue destruction. The present study reveals a great deal of variability between subjects for these three tests, with some having very effective antibody for some functional tests and almost no activity in other tests. The overall results show a close correlation between the production of superoxide and ingestion of *P. gingivalis*, but little few parallels between these two tests and direct killing by serum. This suggests that there may be at least two seperate subsets of immunoglobulin operating in the host.

The immunized group routinely exhibited a greater functional response than the natural and ligature groups; however, the response was not proportional to the titer of antibody present. This result appears to be consistent with the

results of other studies. The functional tests reported in this study should help to form a firm foundation for future studies analyzing the complicated system known as the immune response and it significance in periodontitis.

APPENDIX A1

Complement Fixation Test
Reagents

$MgCl_2$-$CaCl_2$ Stock Solution 10 ml
    2.03 g $MgCl_2$ $6H_2O$ (J.T. Baker Inc. Phillipsburg, NJ)
    0.44g $CaCl_2$ Anhydrous (J. T. Baker Inc. Phillipsburg, NJ)
    Bring to total volume of 10 ml with distilled water

Stock Barbital-buffered Diluent 100 ml
    50 ml distilled water
    4.15 g NaCl (J. T. Baker Inc. Phillipsburg, NJ)
    0.51 g sodium barbiturate (Barbital Sodium C-IV, Fisher Scientific Co. Fair Lawn, NJ)
    Mix until dissolved
    1.7 ml of 1 N HCl mix by swirling
    0.25 ml stock $MgCl_2$-$CaCl_2$ solution
    Bring total volume to 100 ml with distilled water
    (Check pH by creating a 1:5 dilution by adding 4ml distilled water to 1 ml of stock solution. The pH must be 7.3-7.4.)

Gelatin Water 400 ml
    0.5 g gelatin dissolved in 200 ml of distilled water
    Bring mixture to a boil to completely dissolve the gelatin
    Allow to cool and bring total volume to 400 ml with distilled water

Working Barbital-buffered Diluent (BBD)
    75 ml of stock Barbital-buffered Diluent stock solution
    300 ml of gelatin solution
    Mix thoroughly without causing bubbles
    Check pH, must be 7.3-7.4

APPENDIX A2

Complement Fixation Test
Preparation of Sheep Red Blood Cells

The complement fixation test requires sheep red blood cells (SRBC) that have been stabilized in Alsever's solution (Accurate Chemicals and Scientific Corporation, Westbury, NY). The SRBC require the following treatment prior to use in the assay.

1. Aliquot 5 ml of 50% SRBC in Alsever's solution into a graduated centrifuge vial.

2. Centrifuge at 600 x g for 5 minutes at 4°C (Sorvall RT6000B Refrigerated Centrifuge, Dupont).

3. Remove supernatant with a micropipet, leaving SRBC in the bottom of the tube.

4. Add cold Barbital-buffered diluent (BBD) to a total volume of 5 ml.

5. Mix gently to resuspend the cells and centrifuge at 600 x g for 5 minutes at 4°C.

6. Remove supernantant. (Note: the supernatant should be colorless, showing that the SRBCs are stable enough for use.)

7. Resuspend the cells in enough cold BBD to bring the solution to an 8.4% solution. (This should be a total volume of 29.76 ml if the original 50% SRBC in Alsever's solution was well suspended.)

8. The cells are now ready for use.

Appendix A3

Preparation of Complement and Storage

Rabbit Complement

Rabbit complement HLA-A, B and C Typing (Pel-Freeze, Brown Deer, WI) was received in a freeze-dried state and was reconstituted with an equal volume of distilled water (1 ml).

Human Complement

Human complement commercially available (Sera, Complement 39 CH50 units/ml, Sigma) was received in a freeze-dried state and reconstituted with an equal volume of cold deionized water (1 ml).

Human complement from human donors was prepared as follows. Blood was drawn by venipuncture into red topped tubes and allowed to clot. It was allowed to set at room temperature for 30 minutes and then stored at 0°C for 60 minutes. The specimen was centrifuged at 1875 x g for 10 minutes (Sorvall RT6000B) at 4°C. The serum containing complement was removed, aliquoted into 200 µl amounts and stored in 250 µl plastic centrifuge tubes (35 mm L x 6 mm dia, Cole-Parmer Instrument Company, IL). Three human donors participated in the initial donation of serum.

Storage

All complement sources either fresh or reconstituted were subsequently stored at -70°C. When required, the sera were thawed just prior to use. The

fresh human samples were quantitated initially with the complement fixation test and were then tested again after 8 months of -70°C storage to insure that the complement activity had not decreased.

Appendix A4

Preparation of a Color Standard for Complement Fixation Test

A colormetric standard is needed to assess the percent of hemolysis that occurs during the complement fixation test. This standard is prepared as follows:

Preparation of a Hemoglobin solution
1. Add 1.0 ml of a well mixed 8.4% sheep red blood cell suspension to a test tube (15 x 125 mm).
2. Add 7.0 ml of distilled water, and shake the tube until all erythrocytes are lysed.
3. Add 2.0 ml of stock barbital-buffered solution.
4. Mix the hemoglobin solution thoroughly.

Preparation of a 0.84% erythrocyte suspension
1. Resuspend the erythrocytes.
2. Add 1 ml of the 8.4% suspension to a test tube (15 x 125 mm).
3. Add 9.0 ml of cold barbital-buffered diluent to the tube with the erythrocytes.
4. Mix the suspension gently in the tube.

Preparation of the color standards.
1. Eleven tubes (13 x 100 mm) are labelled with the percentage of hemolysis as seen the following table.
2. The appropriate hemoglobin and 0.84% erythrocyte solutions are added following the amounts listed below.
3. Mix the standards gently.
4. Centrifuge the tubes at 600 x g for 5 minutes.
5. Remove the tubes and read the absorbance at 540 nm.
6. Plot the results of known hemolysis and absorbance on a graph (Figure 39).

% Hemolysis	Hemoglobin Solution (ml)	0.84% cell suspension (ml)
0	0.0	1.0
10	0.1	0.9
20	0.2	0.8
30	0.3	0.7
40	0.4	0.6
50	0.5	0.5
60	0.6	0.4
70	0.7	0.3
80	0.8	0.2
90	0.9	0.1
100	1.0	0.0

Figure 39 Hemoglobin Color Standard at 540 nm. The absorbance recorded for a known percentage of sheep red blood cell lysis at 540 nm. The y-axis represents the percentage of sheep red blood cell hemolysis. The x-axis represents the absorbance recorded for a solution of 8.4% sheep red blood cells.
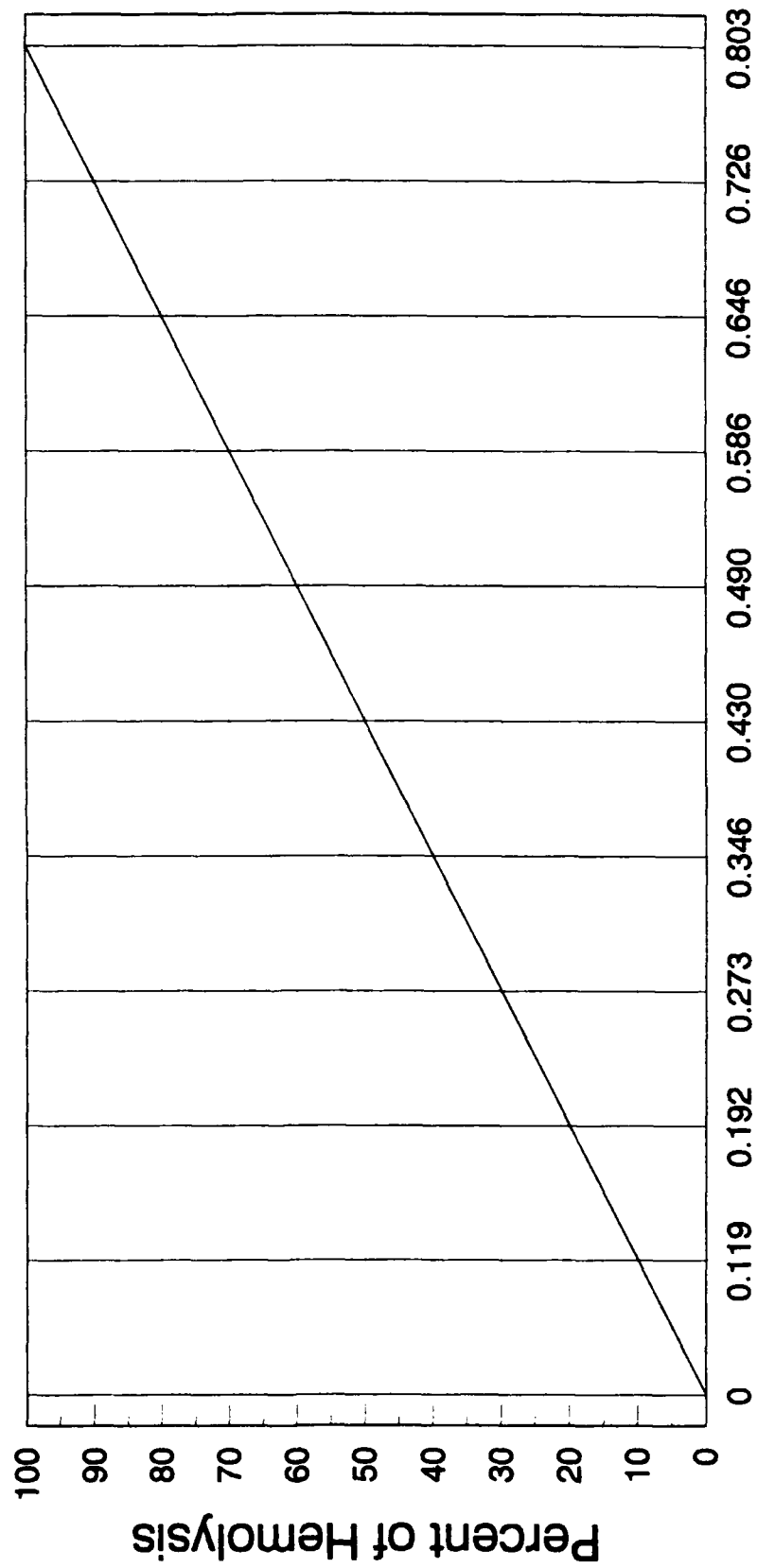
Hemoglobin Color Standard
at 540 nm

Percent hemolysis determined with 8.4% SRBCs

APPENDIX A5

Preparation of Sensitized Sheep Red Blood Cells

The complement fixation test requires that the sheep red blood cells (SRBC) be opsinized with hemolysin using the following procedure.

1. After determination of the optimal hemolysin concentration the appropriate dilution (1:1500) is prepared using barbital-buffered diluent.

2. Approximately 3 ml of sensitized SRBC are prepared for determination of the appropriate complement titer of a serum. This sensitized suspension consists of 50% 8.4% SRBC suspension and 50% hemolysin (1:1500) solution.

3. In order to avoid premature lysis of the SRBC, the SRBC are added to the glass tubes (12 x 75) first, followed by the hemolysin which is added while vortexing the tube at setting of 1.

4. Incubate the mixture for 15 minutes in a 37°C water bath.

5. The SRBC are now ready for assay.

Appendix B

Enzyme Linked Immunosorbent Assay (ELISA)

The following procedure was used to determine the relative amounts of IgG specific to
P. gingivalis 3079.03.

ELISA Procedure:

1. Suspend formalinized whole cell P.g. 3079.03 in Buffer I so the O.D. is 0.3.
   Add 200 µl/well and incubate 3.5 hours at 37°C. Store at 4°C.
2. Wash plate 3x with Buffer II.
3. Add 100 µl of appropriate dilution (diluted in Buffer III) of test serum (rabbit or
   human) to the wells.
4. Incubate 2 hours at room temperature on rotator.
5. Wash plate 3x with Buffer II.
6. Add 100 µl of 1:1000 dilution of Goat anti-human IgG antisera (diluted in
   Buffer III) to wells.
7. Incubate for 2 hours at room temperature on rotator.
8. Wash plate 3x with Buffer II.
9. Add 100 µl of Rabbit anti-goat IgG conjugated to alkaline phosphatase 1:1000
   (dilluted in Buffer III) to the wells.
10. Incubate overnight at room temperature on the rotator.
11. Wash plate 3x with Buffer II.
12. Add 200 µl substrate diluted to 1 mg NNP/ml in Buffer IV. Reaction should
    be completed within 30 minutes at room temperature.
13. Stop the reaction with 100 µl of Buffer V.
14. Read plate at 405-410 nm (Biomek1000, Beckman).

Reagents:

Alkaline phsphatase conjugate: Sigma #A7650 Rabbit anti-goat IgG, heavy and light
        chain specific, affinity purified, alkaline phosphatase conjucated.

p-Nitrophenyl phosphate (NNP): Sigma 104 #144-0

Affinitiy purified antiseras: Goat anti-human IgG antisera, affinity purified. Calbiochem
        #401441 IgG

Stock buffers:

0.2M Sodium carbonate (21.2 g sodium carbonate/1000 ml distilled water)

0.2M Sodium bicarbonate (16.8 g sodium bicarbonate/1000 ml distilled water)

10% Sodium azide (10 g sodium azide/100 ml distilled water)

10X PBS (108 g sodium phosphate dibasic, 11 g sodium phosphate monobasic, 350 g
sodium chloride, bring to 4 L with distilled water and pH 6.96)

Buffer I: For coating plates with antigen pH 9.6
              80 ml sodium carbonate stock
              170 ml sodium bicarbonate stock
              1 ml sodium azide
              bring to 500 ml with distilled water

Buffer II: For washing between steps
              27 g sodium chloride
              1.5 ml Tween 20
              bring to 3 L with distilled water

Buffer III: For diluting samples and antisera
              300 ml 10X PBS
              1.5 ml Tween 20
              6 ml 10% sodium azide
              bring to 3 L with distilled water

Buffer IV: For dissolving NPP substrate pH 9.8
              55 ml sodium carbonate stock
              70 ml sodium bicarbonate stock
              100 mg magnesium chloride
              bring to 500 ml with distilled water

Buffer V: For stopping reaction 1N NaOH
              20 g sodium hydroxide
              bring to 500 ml with distilled water

Plates: Linbro Titertek microtiter plates (#76-301-05, plate sealers #76-401-05).

Appendix C

Fluorescent Filtration: Vanox T (Olympus) Microscope

Excitation	Excitation light wavelength (high pressure mercury burner Hg)	Observation light	Example of application
U (Ultra-Violet)	330 ~ 380 nm (365 nm)	420 nm and up	DAPI
V (Violet)	395 ~ 415 nm (405 nm)	455 nm and up	DAPI used with cyanoacrylate
B (Blue)	380 ~ 490 nm (405 nm, 436 nm)	515 nm and up	
G (Green)	465 ~ 550 nm (546 nm)	590 nm and up	Propidium Iodide

Appendix D

Reagents for Neutrophil Assays

Reagents for preparation of human neutrophils, superoxide anion assay and neutrophil phagocytic assay.

Glucose 0.1 M
      18.01 g glucose
      1 L dH$_2$O
      Filter sterilize
      Store at -20°C
Saline 0.9%
      9 g sodium chloride
      1 L dH$_2$O
      Filter sterilized
      Store at room temperature
Calcium Chloride
      0.11 M calcium chloride
      3.23 g/200 ml H$_2$O
      Store at 4°C
Magnesium sulfate
      0.15M magnesium sulphate
      7.39 g/200 ml H$_2$O
      Store at 4°C
6% Dextran
      60 g Dextran T500 to 1 L of 0.9% saline
      Filter sterilize
      Store at 4°C
20X Saline
      18 g sodium chloride
      100 ml dH$_2$0
      Filter sterilize
      Store at 4°C

Sodium Phosphate Buffer
     0.1M Na_2HPO_4 (Dibasic) 14.2 g/1L dH_2O
     0.1M NaH_2PO_4 (Monobasic) 12.0 g/1L dH_2O
     Make 500 ml(0.1M) monobasic (6.0 g/500 ml dH_2O)
     Make 3L (0.1M) dibasic (42.6 g/3L dH_2O)
     Add the 500 ml monobasic to 4L bucket (pH 7.4) while stirring add dibasic slowly
     until pH 7.4 (about 2.5L)
     Filter sterilize
     Store at 4°C.

Phosphate Buffered Saline (PBS)
     100 ml 0.9% saline
     12 ml sodium phosphate buffer
     4 ml potassium chloride
     pH to 7.4 with NaOH
     Store on ice

Krebs Ringer Phosphate plus Glucose
     30 ml PBS
     250µl Calcium chloride solution
     250µl Magnesium sulfate solution
     1.5 ml glucose

Cytochrome C
     0.5 g cytochrome C (Sigma)
     40 ml PBS
     Filter sterilze
     Store frozen

Superoxide Dismutase
     1mg/ml H_2O Superoxide dismutase (Sigma)
     Filter sterilize
     Store at 4°C

Cytochalasin B (Sigma)
     1 mg cytochalsin B
     1 ml DMSO
     Store at 4°C

Acid Citrate Dextrose (ACD)
     8g citric acid
     22 g sodium citrate
     24.5 g dextrose
     Total volume to 1000ml dH_2O
     Filter sterilize
     Store frozen at -20°C

N-ethylmaleimide(NEM)
     0.01M
     Store at 4°C

Ficoll-Paque (Pharmacia LKB, Biotechnilogy AB, Sweden)
     Store at 4°C

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VITA

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